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Affective Responses in Cocaine-experienced Rats Reveal Cue-induced Drug Craving and Cocaine Reward Magnitude

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This dissertation is dedicated to Courtney Paige Griffin.

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The development and persistence of cocaine dependence are greatly influenced by emotional affect and cocaine associative learning. Cocaine is known to enhance nucleus accumbens (NAcc) dopamine, serve as a positive reinforcer and produce negative effects, such as anxiety that may influence cocaine intake behavior. In the first study, I investigated the effects of the anxiolytic, diazepam on NAcc dopamine levels and cocaine self-administration behavior. These are two factors associated with cocaine rewarding effects. Diazepam has no effect on NAcc dopamine, but affects cocaine self-administration. This supports the notion that decreasing the anxiogenic effects of cocaine increases the rewarding value in a dopamine independent manner. Therefore, increasing the aversive effects of cocaine might be a novel approach to fight cocaine dependence. In the second study, I studied cocaine-induced associative learning and changes in affect during cocaine conditioning and extinction. 50-kHz ultrasonic vocalizations (USVs) in rats are thought to reflect positive affect and occur upon appetitive stimuli and with cocaine delivery. First, I explored whether USVs might be elicited in anticipation of impending drug delivery. Shortly into conditioning, rats elicited USVs when placed in the cocaine-associated environment. USVs progressively increased, indicating a growing

learned association between cocaine intake and cocaine-associated cues. This suggests that USVs may be a useful model for investigating cocaine craving and serve as a pharmacological target for interventions aimed to reduce cocaine craving and relapse. I then examined the effects of short-term deprivation of cocaine and cocaine cues on cocaine-conditioned USVs, which were both exaggerated after abstinence. The results may have clinical implications, in that intermittently avoiding cues or context may enhance drug cue salience and increase the probability of relapse. Motivational aspects of cocaine were assessed comparing commonly measured lever response rate and locomotion with cocaine-induced USVs during cocaine administration and extinction. In agreement with prevailing findings, lever responding for cocaine and cocaine-induced locomotor activity increased across conditioning sessions. However, the number of USVs evoked in response to cocaine infusion decreased with cocaine experience. These findings suggest growing tolerance to the rewarding properties of cocaine. These studies underscore the value of USV assessment during drug dependence studies.

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Chapter 1: Background

COCAINE

History of Cocaine

Cocaine is an alkaloid derived from Erythroxylon coca. For thousands of years, indigenous South Americans have grown and harvested coca leaves and chewed them to generate their properties. Besides their nutritious value, the effects of extracted cocaine were mainly used for trances in religious ceremonies and to regain strength and energy during work in high altitudes. The Spanish conquerors in the 16th century failed to ban the chewing of coca leaves since the energizing effects were crucial for the workers. Instead, the performance-enhancing effects were quickly acknowledged and coca leaves were introduced to Europe (Gootenberg 1999). In 1855 the German chemist Friedrich Gaedcke was the first to isolate the active compound of the coca leave and called it "erythroxyline" (Gaedcke 1855). The dissertation work performed by the German PhD student Albert Niemann lead to the purification process of "cocaine" in 1860 (Niemann 1860). Even though the synthesis of cocaine is possible (Huisgen 1961), cocaine is up to date processed directly out of the coca leaves mainly grown in South America.

The first commercial beverage containing cocaine was the wine "Vin Mariani". This beverage was endorsed by Pope Leo XIII, and its creator was awarded the "Vatican Gold Metal". Vin Mariani was imitated by the American pharmacist John Pemberton who, after the Prohibition period, was forced to remove its alcohol content and created the still famous soda drink Coca-Cola® (Worldpress 2011). Whether Coca-Cola® has ever contained cocaine is controversial, and the original recipe is secretly kept at the

company's headquarters in Atlanta, GA. Interestingly, a radio program recently claimed to have discovered the original recipe showing coca as an ingredient (Life 2011). Since cocaine has been shown to be highly addictive, cocaine and its products were banned from the US market, and following the Controlled Substances Act of 1970 cocaine is currently listed as a Schedule II drug. Drugs in this category have a high abuse potential, may cause severe psychological or physical dependence, but are accepted for medical treatments in the United States. Cocaine's medical use is limited to its topical local anesthetic and vasoconstrictive effects (reduced bleeding) during nose, mouth and throat surgeries. The chemical structure of cocaine served as the basis for the development of today's local anesthetics.

Mechanism of Action of Cocaine

Cocaine is typically seen as an indirect dopamine agonist via dopamine transporter (DAT) inhibition (Carroll et al. 1992; Heikkila et al. 1975), with a binding site different than the substrate-binding site (Wayment et al. 1998). However, cocaine is not DAT-specific; it also blocks other plasma membrane transporters, such as the serotonin and norepinephrine transporter (Chen et al. 1997; Chen and Reith 2002). These transporters are located at the nerve terminals and move previously released substrates back into the neuron. The drive behind the reuptake is a concentration gradient of sodium ions (Chen et al. 2004). Cocaine binding inhibits the symport of one substrate, one chloride and two sodium ions through the transporter. Since reuptake is the major inactivation mechanism for monoamines in the synaptic space (Horn 1990) all three monoamine levels rise due to cocaine-induced transporter inhibition (Heikkila et al. 1975; Ross and Renyi 1967).

Several *in-vivo* microdialysis studies have shown that cocaine increases extracellular dopamine levels in the NAcc (D'Souza and Duvauchelle 2006; Hemby et al. 1997; Ikegami and Duvauchelle 2004; Ferris et al. 2011; Pettit and Justice 1991), the medial prefrontal cortex (Ikegami and Duvauchelle 2004; Sorg et al. 1997) and the dorsal striatum (D'Souza and Duvauchelle 2006). This was found to be dose-dependent for the NAcc (Pettit and Justice 1991). However, the discovery of genes for the DAT transporters and the consequent creation of mice lacking one or more transporters questioned the once well-established exclusive involvement of DAT in eliciting the reinforcing properties of cocaine (Ritz et al. 1987; Ritz et al. 1988; Kuhar 1992; Segal and Kuczenski 1987; Wise 1996). However, up to date, researchers agree on the involvement of enhanced dopamine activity and its subsequent neuroadaptations in cocaine reinforcement and the development of cocaine dependence [see reviews: e.g., (Torres et al. 2003; Uhl et al. 2002)].

Peripheral effects of cocaine (e.g., increased heart rate and blood pressure) are attributed to enhanced levels of synaptic norepinephrine in terminals of the sympathetic nervous system (Foltin et al. 1995; Resnick et al. 1977).

Cocaine Effects in Humans

Sigmund Freud might be one of the most famous cocaine users. Back in the 1880's, he stated the effects of cocaine as follows:

> You perceive an increase of self-control, possess more vitality and capacity for work. This result is enjoyed without any of the unpleasant aftermaths, which accompany exhilaration through alcoholic means (Byck 1974).

As described by many cocaine abusers, small amounts of cocaine lead to euphoria, revitalize, energize, and create talkativeness and increase mental alertness.

However, chronic cocaine use leads to a decline in its pleasurable euphoric effects, resulting in an increase in frequency of cocaine use and the administration of escalating cocaine doses. Soon, also Freud discovered the danger of cocaine use:

> In 1885 it was I who had recommended the use of cocaine, and I had been gravely reproached in consequence. A dear friend, who had died […] had hastened his end by the misuse of this remedy. […] I had recommended the remedy for internal use only during the withdrawal of morphia; but he immediately gave himself injections of cocaine (Byck 1974).

Furthermore, increased doses of cocaine eventually lead to the desired "high", but are usually accompanied by feelings of irritability, restlessness, anxiety and paranoia.

Animal studies suggest that dopamine plays a major role in the induction of cocaine's rewarding effects. However, up to 1996, this has not been verified for the human brain. A positron emission tomography (PET) study performed by Volkow and colleagues finally supported the importance of dopamine activity in cocaine-induced reward in humans. Using the "cocaine-like psychostimulant" methylphenidate (MP) (Wang et al. 1997), which is commonly prescribed for attention deficit disorder in children (e.g., Ritalin®) (Byck 1974), cocaine has been shown to increase striatal dopamine activity through DAT inhibition (Volkow et al. 1996; Volkow et al. 1999). This increase in dopamine activity is correlated with the subjective feelings of " high" and "rush". Importantly, these studies were performed on healthy subjects. PET studies comparing the effects of MP on healthy individuals and cocaine-dependent subjects revealed that cocaine dependents show lower dopamine activity in the brain and experience a decreased "high" and "restlessness" (Volkow et al. 1997). In addition, cocaine-dependent subjects rate cocaine craving higher (Volkow et al. 1997).

Since cocaine associations have been shown to be important in the development as well as persistence of cocaine dependence, Ehrman and colleagues (1992) investigated

the effects of conditioned stimuli in cocaine abusers. They showed that the subjects elicit conditioned responses (e.g., physiological, such as decrease in skin temperature or increase in heart rate; emotional, such as cocaine craving and withdrawal) to cocaine-related stimuli, but not to neutral stimuli or stimuli picturing unacquainted drugs (Ehrman et al. 1992). PET studies have linked this cue-induced cocaine craving to elevated dopamine activity in the dorsal striatum, which is thought to be involved in habit formation (Volkow et al. 2006; Wong et al. 2006). Similarly, this increase in dopamine activity is correlated with ratings of cocaine craving in conjunction with cocaine cues (Volkow et al. 2006; Volkow et al. 2008).

DRUG DEPENDENCE

The National Institute on Drug Abuse (NIDA), defines addiction or drug dependence as " […] a chronic, relapsing brain disease that is characterized by compulsive drug seeking and use, despite harmful consequences". This brain disease is thought to develop after single or frequent drug abuse. A study using data from the U.S. National Comorbidity Survey (pooled 1990-1992) reported that a maximum of 16% of cocaine abusers develop cocaine dependence within 10 years of first exposure (Wagner and Anthony 2002). This raises the question: why does not everyone who uses drugs become dependent? It is well established that all drugs of abuse affect specific areas of the brain that are involved in natural reward, learning and motivation (Everitt et al. 2008; Koob 2009). It is postulated that these drug effects cause changes in normal brain reward activity, which lead to neuroadaptations and eventually drug dependence.

Theories of Drug Dependence

In Solomon's Opponent Process Theory of Addiction, drug-induced neuroadaptations are thought to achieve homeostasis (Solomon 1980; Solomon and Corbit 1974). According to this theory, drugs of abuse strongly activate brain reward circuits, and consequently, the brain develops mechanisms to overcome this over-stimulation. Since the latter process is associated with withdrawal effects of the drug, the combination of these two opposing processes leads to either pleasant or unpleasant overall effects. Over time the overall drug effect shifts towards the unpleasant side, as the neuroadaptations have grown stronger. Solomon's theory entails the development of tolerance to the pleasant drug effects, which explains escalated drug intake longing for the wanted high. Increased drug intake results in further neuroadaptations, which eventually leads to drug dependence. A modification of this theory by Koob and colleagues proposes a potential allostatic state (caused by neuroadaptations to achieve former homeostasis), which leads to the manifestation of an "anti-reward" system that drives withdrawal (i.e., "dark-side of addiction") and subsequent relapse (Gracy et al. 2001; Koob and Le Moal 2005).

The second theory of drug dependence first proposed by Robinson and Berridge in 1993 states that drug-induced neuroadaptations cause the development of sensitization to certain drug effects, as well as to stimuli associated with the drug (Robinson and Berridge 1993; Robinson and Berridge 2008; Robinson and Berridge 2003). The Incentive-Sensitization Theory of Addiction states that processes regulating normal motivated behavior to reward and reward-associated stimuli (incentive salience) are sensitized due to drug-induced neuroadaptations. This sensitization of incentive salience to drugs and drug-associated stimuli (i.e., "incentive sensitization"), a pathological

motivation for the drug, leads to a persistent and dramatic increase in drug 'wanting', compulsive drug intake, and relapse of abstinent patients.

An emerging view supported by a compilation of studies, implies that drug-induced neuroadaptations cause abnormal learning, which leads to the formation of strong drug-stimulus and drug-response associations. These acquired over-learned associations predict the pleasurable outcome of drug consumption and are thought to result in habit formations leading to drug-taking behaviors that become habitual, automated and eventually compulsive drug consumption (Di Chiara 1999; Everitt et al. 2008; Everitt and Robbins 2005; O'Brien et al. 1992; Tiffany 1990; White 1996).

Drug Reinforcement and Reward

Specific areas of the brain involved in reinforcement have been first identified by Olds and Milner in 1954 (Olds and Milner 1954). They discovered that animals readily self-administer electrical stimulations into the septal area of the brain. A decade later, Hillarp and colleagues showed that these areas consist of dopamine neurons projecting from the ventral mesencephalon to cortical and striatal regions (Hillarp et al. 1966). These dopaminergic projections are thought to be responsible for psychostimulant-induced locomotor effects and instrumental conditioning (Anlezark et al. 1971). A review by Kornetsky and Bain (1992) discusses brain stimulation studies and concludes that all drugs of abuse activate a reward system that projects from the ventral tegmental area (VTA) to the limbic and mesocortical systems of the brain. Since the discovery of the brain reward system (i.e., mesolimbic dopamine system), there is growing evidence that enhanced nucleus accumbens (NAcc) dopamine activity plays a key role in the *acute* rewarding, reinforcing and motivating properties of drugs of abuse. However, *chronic*

drug abuse initiates neuroadaptations in the brain, which eventually leads to drug dependence in some individuals. Drugs of abuse (e.g., cocaine, amphetamine, opiates and nicotine) elicit their rewarding effects through different sites of action in the brain reward system. However, all of these effects cause an increase of extracellular NAcc dopamine (Koob 1992). For example, stimulants, such as cocaine and amphetamine act on dopamine transporters (DAT) of dopamine neurons projecting from the VTA, and hereby increase NAcc dopamine (D'Souza and Duvauchelle 2006; Ferris et al. 2011; Hemby et al. 1997; Ikegami and Duvauchelle 2004; Pettit and Justice 1991). Opiates directly act on endogenous opioid receptors located on gamma-aminobutyric acid (GABA) interneurons in the VTA. This causes an inhibition of an inhibitory GABA effect on efferent VTA dopamine neurons to the NAcc, resulting in a net activation of these VTA neurons, thus leading to an increased dopamine neurotransmission in the NAcc (Xi and Stein 2002). Similarly, nicotine increases firing rates of VTA dopamine neurons by acting on nicotinic acetylcholine receptors in the VTA (Corrigall et al. 1994; Pidoplichko et al. 1997). Interestingly, even though all drugs of abuse enhance extracellular NAcc dopamine, not all their rewarding or reinforcing properties seem to depend on increased NAcc dopamine activity. For example, it has been shown that heroin self-administration is not affected by the pretreatment with a dopamine antagonist (Ettenberg et al. 1982). However, studies on psychostimulants, such as cocaine and amphetamine support the theory that the mesolimbic dopamine system (i.e., dopamine neurons connecting VTA and NAcc) plays an important role in drug reward. Systemically (e.g., flupenthixol, pimozide) or directly into the NAcc (SCH23390) administered low doses of dopamine receptor antagonists result in augmentation of cocaine-self-administration on a fixed-ratio (FR) schedule of reinforcement (De Wit and Wise 1977; Ettenberg et al. 1982; Maldonado et al. 1993; McGregor and Roberts 1993) (see ‘Animal Models of Cocaine Dependence’ for the

description of different schedules of reinforcement). These studies suggest that increased cocaine intake compensates for dopamine receptor antagonism, which ultimately produces reward. Consequently, lever responding is reinforced. Additionally, the reduction of cocaine reward by dopamine antagonists has been supported by a study using self-administration on a progressive ratio schedule. Pretreatment of systemic haloperidol (Roberts et al. 1989) or intracranial injections of SCH23390 (McGregor and Roberts 1993) reduces the effort (i.e., breakpoint reduction) to obtain another cocaine dose. Furthermore, the administration of pimozide blocks the development of conditioning to a cocaine environment (Beninger and Herz 1986; Morency and Beninger 1986), the rats do not experience cocaine as rewarding and do not show preference for the cocaine-paired environment. Additionally, disruption of NAcc dopamine activity leads to a decrease in cocaine reward. High doses of systemic flupenthixol (Ettenberg et al. 1982), pimozide (De Wit and Wise 1977) or 6-hydroxydopamine-induced lesions of the NAcc (Roberts et al. 1977; Roberts et al. 1980) cause an abrupt decrease in FR cocaine self-administration (i.e., reinforcing properties). Interestingly, many animals in the NAcc lesion study timely resume lever responding for cocaine, which is correlated with undestroyed NAcc dopamine content (Roberts et al. 1980). It is necessary to mention that dopamine lesion in the striatum can lead to motor-impairments [see review (Schwarting and Huston 1996)], that could lead to a reduction of self-administration behavior. However, motor-impairments were not observed in this particular study (Roberts et al. 1980).

Research in the last two decades has proven that other dopamine systems also play a role in the acute rewarding properties of drugs of abuse. These include the nigrostriatal (i.e., dopamine neurons connecting substantia nigra and dorsal striatum) and

the mesocortical system (i.e., dopamine neurons connecting VTA and prefrontal cortex) (Kornetsky and Bain 1992; Volkow et al. 2011; Wise 2009).

Dysregulation of Reward and Allostasis

According to Koob and colleagues, frequent drug abuse leads to tolerance in the brain reward system and sensitization in the anti-reward system, which is manifested in withdrawal effects and an overall negative affective state (i.e., anhedonia) (Koob and Le Moal 2005; Koob and Le Moal 1997). A decrease of basal NAcc dopamine levels of animals allowed to self-administer cocaine (unlimited access) is apparent for 12 hours after discontinuation of cocaine access (Weiss et al. 1992). Interestingly, this decrease in extracellular NAcc dopamine is positively correlated with the length of previous cocaine access (Weiss et al. 1992). In a different approach, animals show an increase in thresholds of intracranial self-stimulation in the medial forebrain bundle after extended cocaine self-administration sessions were terminated (Markou and Koob 1991). Similarly, the elevation of threshold for intracranial stimulation is positively correlated with previous cocaine intake (Markou and Koob 1991).

In all individuals, homeostasis is necessary to maintain survival, such as the regulation of blood glucose levels to sustain energy (Cannon 1929). However, the regulatory capacity of homeostasis seems inefficient and adaptations to changes in physiological parameters produce a stronger counter-balanced reaction, known as allostasis (Sterling 2011). Thus, allostasis leads to the achievement of stability in unstable organisms, and in healthy individuals, allostasis is shut off after stability is attained (McEwen 1998; Schulkin 2003). However, frequent activation of allostatic systems leads to their over-stimulation and eventually malperformance. Regarding the Salomon's

opponent-process theory it can be speculated that the attenuation of the rewarding effects of drugs of abuse is due to an overstimulated allostasis (i.e., allostatic state) initiated by frequent drug intake. In case of frequent drug abuse, it is suggested that allostasis leads to the recruitment of other neurotransmitter and hormonal systems to achieve normal reward levels (Koob and Le Moal 2001). One of these systems is the brain stress system, which is significantly regulated by the corticotropin-releasing hormone (CRH). Long-access self-administration of cocaine attenuates CRH release in the amygdala (75%), but during following acute withdrawal, amygdala CRH levels rise up to 400% (Richter and Weiss 1999). Since increased CRH levels are correlated with negative affect (Richter and Weiss 1999), its increase explains the anhedonic state during cocaine withdrawal. It has been reported that abnormal brain stress system activity can continue long after drug administration ended (Kreek and Koob 1998) and that intracranial pretreatments with CRH antagonist (i.e., immunoserum) during cocaine administration block anxiety-like behavior of the animals during withdrawal (Sarnyai et al. 1995). The direct involvement of cocaine-induced increased dopamine levels in CRH changes has been supported by lesion studies of the mesolimbic/mesocortical dopamine system (Goeders et al. 1990). Therefore, cocaine-induced enhancement of dopamine activity in the brain reward system leads to the activation of the brain stress system during abstinence. This allostatic brain response is mirrored in the development of withdrawal symptoms, such as anxiety and anhedonia, which leads to escalated drug intake to alleviate these symptoms. However, the dysregulation theory might not be accountable for the persistenc in relapse.

Associative Learning

The Incentive-Sensitization Theory of Addiction, as well as the abnormal learning theory imply that learning is an important factor in the development and persistence of drug dependence (Robinson and Berridge 1993; Robinson and Berridge 2008; Robinson and Berridge 2003). Either compulsive drug wanting (O'Brien et al. 1998; Robinson and Berridge 2008) or drug intake (Everitt et al. 2008) can be triggered by acquired drug-cue associations. Two dissociable processes are known to be involved in learning these drug-induced associations: Pavlovian conditioning and instrumental or operant conditioning (Wassum et al. 2011). Pavlov was interested in natural reinforcers (unconditioned stimulus), such as food, that cause a reflex or unconditioned response, such as salivation. After pairing the unconditioned stimulus with a neutral stimulus (e.g., sound or light), the neutral stimulus alone evokes salivation, termed "psychical reflex" or "conditioned response" by Pavlov (Pavlov 1927). The presence of the reinforcer is important to maintain the conditioned response, or else, it extinguishes. Influenced by Pavlov, the first fully functional operant conditioning chamber was presented by Skinner in 1938 (Skinner 1938). In contrast to the non-contingent Pavlovian conditioning, animals learn to "operate" (e.g., lever response, nose poke) for delivery of the reinforcer. Since the animal contingently operates to obtain reinforcers, the operant behavior (response) and not the conditioned stimulus (i.e., Pavlovian conditioning) becomes associated with the reinforcer and strengthens over time. In animal studies, cues associated with cocaine intake lead to an enhancement of cocaine-induced behaviors (e.g., lever response) next to a persistence in drug-seeking after extinction (Di Ciano and Everitt 2002; Duvauchelle et al. 2000; Meil and See 1996; Panlilio et al. 1996; Weiss et al. 2000; Weiss et al. 2001).

Repetitive drug administration can have multiple outcomes on drug effects. It can have no effect, attenuate (i.e., development of tolerance), or enhance (i.e., development of

sensitization) drug responses. It has been suggested that sensitization to psychostimulant effects can be induced by drug context (Robinson and Berridge 1993; Robinson and Berridge 2003; Robinson et al. 1998). This phenomenon might be the basis for the development of strong drug-stimulus or drug-response associations. This is of special interest since behavioral sensitization following psychostimulant administration has been shown to be persistent over years (Castner and Goldman-Rakic 1999), which might explain the persistence in drug associations leading to drug craving and relapse. For example, animals receiving cocaine and saline injections in two different contexts show behavioral sensitization effects only when the test context was previously paired with cocaine (Badiani et al. 1995). Furthermore, rats injected with saline in the previously cocaine-paired environment elicit a context-induced behavioral response, a typical Pavlovian conditioned response to the drug context (Badiani et al. 1995).

It is not clear what develops first: sensitization or learned drug associations. Both drug-induced events have in common that they are maintained by long-lasting neuronal changes. However, it has been shown that cocaine increases glutamate release in the VTA (Kalivas and Duffy 1995) and produces long-term potentiation (LTP) in VTA dopamine neurons (Ungless et al. 2001). The importance of glutamate (i.e., LTP induction) in learning and memory has been widely accepted (Grunwald et al. 1999; Horvitz et al. 2007; Pennartz et al. 2000). In effect, glutamate transmission is thought to be involved in drug-related reward and drug-associative learning (Hyman and Malenka 2001; Kalivas and O'Brien 2008). Overall, the importance of cocaine associative learning in cocaine craving and relapse urges to further investigate the development and persistence of cocaine associations.

Habit Formation

In contrast to its role in associative learning, the role of dopamine in the expression of conditioned responses (e.g., habits) has been challenged. This challenge originated from observations of Parkinson patients. Parkinson disease is a neurodegenerative disease with the loss of dopamine neurons in the nigrostriatal pathway, which results in major motor-impairments. Interestingly, only internally controlled motor movements seem to be impaired, since Parkinson patients show normal motor movements when external stimuli, such as a fire alarm triggers the movement [see review (Jahanshahi and Frith 1998)]. In animal studies, the ability of external stimuli to trigger conditioned responses has also been shown to be dopamine independent. For example, the administration of the dopamine receptor antagonist SCH23390 leads to the disruption of stimulus-paired and stimulus-absent acquisition of goal-directed behavior during early training (Horvitz et al. 2007). However, after acquisition, SCH23390 administration does not affect the well-learned goal-directed behavior, when this is triggered by the associated stimulus (Choi et al. 2005; Horvitz et al. 2007; Horvitz and Eyny 2000). In regard to drug consumption, stimuli that are associated with drug administration have been shown to induce drug craving and compulsive drug intake. "Drug urges" have been proposed to greatly influence this automated behavior (Tiffany 1990), which is

> fast and efficient, readily enabled by particular stimulus configurations […], initiated and completed without intention, difficult to impede in the presence of triggering stimuli, effortless, and enacted in the absence of awareness [see review: (Everitt and Robbins 2005)].

Taken together, habit formation leads to compulsive drug taking behavior, especially when initiated by stimuli associated with the drug. In addition, this automated

behavior is thought to be dopamine independent and therefore seems to be regulated by expected drug reward and not the reward per se.

Relapse

There are several reasons why abstinent drug dependents fail to resist further drug consumption: Firstly, drug seeking is initiated through memories of the acute rewarding effects, such as the anticipated high. Secondly, activation of the "anti-reward" system causes withdrawal effects (Koob and Le Moal 2005). Even though withdrawal is transient, it can be induced by drug associations. Thirdly, relapse can be induced by strong drug cravings initiated by learned drug-cue associations. Interestingly, the necessity of drug craving for the maintenance of compulsive drug intake as well as for relapse have been declared by Jellinek in the mid 1950's and early 60's (Jellinek 1955; Jellinek 1960). Since Jellinek's theory could not be supported by clinical studies, his idea disappeared for about 20 years [see review for details: (Tiffany 1990)]. In the early 1980's his theory revived concurrently with the suggestion that drug associations play a major role in the stimulation of drug craving and relapse.

One of cocaine's effects is the increase of mental alertness. This is expressed in elevated senses like sight, hearing and touch. Those characteristics contribute to an enhanced perception of the drug-taking environment and/or other stimuli under the influence of cocaine. Therefore, the development of strong association between cocaine effects and the drug-taking environment and/or other stimuli is thought to be a major factor in the development and persistence of cocaine dependence and the risk of relapse. It has been reported that the encounter with cocaine-associated stimuli can elicit strong ratings of cocaine craving in cocaine dependent patients (Avants et al. 1995; Childress et

al. 1999; Ehrman et al. 1992; Kilgus and Pumariega 1994; Maas et al. 1998) and accounts for 34% of the cases of abstinent cocaine patients that experienced relapse (Wallace 1989).

ANIMAL MODELS OF COCAINE DEPENDENCE

Currently, there are no effective pharmacological treatments available to "cure" cocaine dependence. However, the mechanisms leading to neuronal changes underlying cocaine dependence have been intensively studied in the last three decades suggesting a few possible medications. This is only possible through the development and use of animal models. Major animal models presently used in drug dependence research include:

1) Conditioned Place Preference (CPP) or Aversion (CPA)

CPP/CPA studies have been intensively used to reflect positive (reward), negative (aversion) or the lack of affective properties of drugs of abuse and to test possible pharmacological treatments for their effectiveness. In more than 50 publications, cocaine has been shown to reliably produce CPP [see review (Calcagnetti et al. 1995)]. In the CPP paradigm, animals receive alternating administrations of cocaine and vehicle in two distinct but unbiased environments. On test day, drug-free animals have access to both environments, and place preference is measured by the amount of time they spent in the cocaine-paired side. Possible pharmacological treatments can affect cocaine-induced CPP by blocking or masking (e.g., being aversive themselves) the rewarding effects of cocaine.

2) Drug Self-Administration

Drug self-administration studies have been used to test the reinforcing properties of a drug of abuse, in other words, how well the drug reinforces associations between a response and an outcome, such as a reward (e.g., instrumental conditioning). Animals only acquire operant responding (e.g., lever response or nose poke) when the drug is rewarding. Reward or reinforcement efficacy can be affected by the route of drug administration. Oral self-administration of cocaine has been shown to be challenging, as it can induce reinforcing efficacy (Falk and Lau 1997; Falk et al. 1996) or not (Bell et al. 1993). However, drinking is established when cocaine is for example introduced in a preferred (e.g., glucose) solution, which is later substituted with water (Falk et al. 1996). However, 460 hits for " intravenous cocaine self-administration" compared to 76 hits for "oral cocaine self-administration" in the pubmed database from 2000-2010 reveals that intravenous administration of cocaine is the main choice of model cocaine for self-administration. Two schedules of reinforcement are mainly used to differentiate between reward efficacy (i.e., fixed ratio, FR) and reward value (i.e., progressive ratio, PR). Animals performing on FR schedules of reinforcement, receive a reward after a fixed number (e.g., either 1, 5, or 10) of operant responses. On PR schedules, responses required for each subsequent reinforcement are progressively increased. The reward value of a drug is measured as the highest number of operant responses performed (i.e., breakpoint) to receive the last drug injection. FR and PR schedules of reinforcement can have different outcomes, but lead to the same conclusion. For example, partial dopamine receptor blockade leads to an increase of operant responses under a FR schedule (De Wit and Wise 1977), presumably because the reward efficacy is compensated by higher cocaine intake. Under a PR schedule, the breakpoint is lower (Roberts et al. 1989)

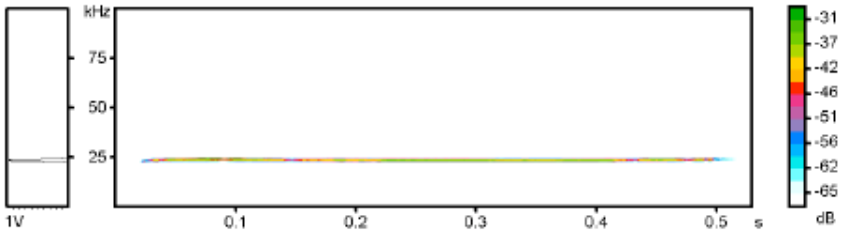
because the effort required for each subsequent lever response is greater than the efficacy of the reduced reward.

CPP and self-administration paradigms have in common that the animals are conditioned to drug-associated stimuli (i.e., CPP: Pavlovian conditioning; self-administration: instrumental conditioning). The major difference between these two paradigms is that animals in CPP studies typically receive experimenter-administered drug injections, whereas animals in the self-administration paradigm the rat's amount of drug consumed is contingent on behavior. In studies investigating these two different modes of administration, a self-administering animal is paired with a yoked animal that receives non-contingent infusions under the same conditions as its operating counterpart. The mode of administration (e.g., self or yoked) influences neurochemical responses to cocaine. For example, it has been shown that rats self-administering cocaine have higher extracellular NAcc dopamine levels than their yoked counterparts (Hemby et al. 1997). Since animal models are thought to mirror human drug intake behavior, the self-administration method seems much more reliable.

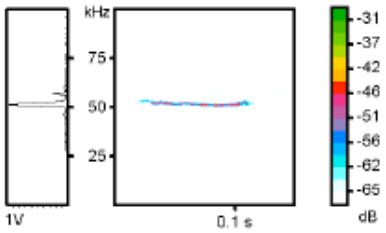
Self-administration paradigms can be further modified to investigate drug-induced phenomena, such as drug-seeking and reinstatement of operant behavior (i.e., relapse). Drug-seeking can be initiated by removing the reinforcer that lead to the acquisition of operant behavior (i.e., extinction). Generally, removal of the reinforcer causes a temporary escalation of operant behavior, which then gradually extinguishes after several sessions. However, if the operant behavior is automated, the behavior might not be completely dependent on the reinforcement outcome, and responses continue to be made (Horvitz 2001).

ULTRASONIC VOCALIZATIONS (USVS) OF RATS

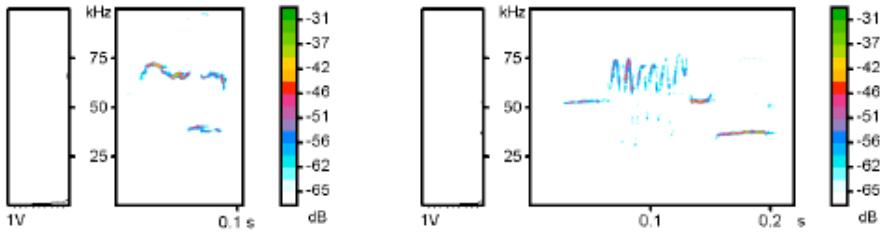
Cocaine-induced emotional responses in laboratory animals have been inferred from changes in mesolimbic dopamine and from specific behaviors of the animals. From these studies it has been reported that increases in mesolimbic dopamine are associated with both, positive as well as negative emotional effects (Kelley and Berridge 2002; Pezze and Feldon 2004). Because of this dichotomy, the interpretation of the outcomes observed in these studies is neither easy nor straightforward (Le Moal and Simon 1991; Wise 2004). Additionally, interpretation of cocaine-induced behavior has also resulted in controversy: for example, an increase in operant behavior that maintains cocaine administration can be interpreted as either a result of the rewarding properties of the drug or as an artifact of the motor-stimulating drug effects. Therefore, ultrasonic vocalizations (USVs) of rats, which have been postulated to represent the emotional state of the animal, have lately received increased attention in drug dependence studies. USVs are regarded as real-time measurements of the emotional states of rats. For example, the presence of a play partner or food presentation has been shown to cause an increase in the emission of high frequency ("50-kHz") calls (Burgdorf et al. 2000; Knutson et al. 1998), whereas the presence of a predator or the touch by an unfamiliar human elicits low frequency ("22-kHz") calls (Blanchard et al. 1991; Brudzynski and Ociepa 1992). It has thus been postulated that the analysis of USVs (based on their frequency) allows distinguishing between the positive and the negative affective state of the animal (Knutson et al. 2002).



Example of a 22-kHz rat USV



Example of a flat 50-kHz rat USV



Examples of frequency modulated 50-kHz rat USVs

In drug dependence studies, short-term (Ahrens et al. 2009; Mu et al. 2009; Wintink and Brudzynski 2001) as well as long-term (Barker et al. 2010; Browning et al. 2011) administrations of amphetamine and cocaine have been shown to increase the emission of positive 50-kHz USVs. In effect, the activation of dopaminergic transmission in the mesolimbic dopamine system by central amphetamine and glutamate administration as well as electrical stimulation of the brain has been shown to increase

the emission of 50-kHz calls (Burgdorf et al. 2001; Burgdorf et al. 2007; Fu and Brudzynski 1994). Furthermore, increased emissions of 50-kHz USVs have been reported in environments associated with morphine, amphetamine and cocaine administration, (Knutson et al. 1999; Ma et al. 2010; Maier et al. 2010). In addition, short-term deprivation of cocaine and cocaine context enhances the emission of anticipatory 50-kHz USVs (Maier et al. 2010). Thus, USVs reflect associative learning between drug and drug-paired environments and might be a novel measurement for cocaine craving that predicts relapse. Cocaine taking behaviors are strongly influenced by the emotional state of the individual as well as manifested cocaine associations. USVs provide a non-invasive and real-time measurement of the emotional and motivational states of rodents during cocaine treatment. USVs do not merely correlate with other common measures, such as locomotor activity or operant behavior, but reveal independent patterns of drug craving and relapse. Most importantly, including USV measurements into drug dependence research will consequently lead to the discovery of medications and treatment strategies for cocaine dependence.

Chapter 2: Diazepam Alters Cocaine Self-Administration, but not Cocaine-Stimulated Locomotion or Nucleus Accumbens Dopamine [1]

ABSTRACT

Cocaine is known to enhance nucleus accumbens dopamine (NAcc DA), serve as a positive reinforcer and produce negative effects, such as anxiety. The influence of diazepam on cocaine intake, cocaine-stimulated behavioral activity and NAcc DA was investigated using self-administration and experimenter-administered intravenous (i.v.) cocaine. In Experiment 1, rats were pretreated with diazepam (0.25 mg/kg) or saline (0.1 ml) 30 minutes prior to 20 daily 1-hr cocaine (0.75 mg/kg/inj) self-administration sessions. Cocaine intake increased for all animals across sessions, but was highest in diazepam-pretreated animals. Diazepam rats also self-administered their first cocaine injection of each session faster than controls. Experiment 2 utilized in vivo microdialysis to assess NAcc DA levels before and after experimenter-administered i.v. cocaine injections (0.75 mg/kg/injection x 2; 10-min interval) in diazepam- and saline-pretreated rats. Group differences were not revealed across basal and cocaine-stimulated NAcc DA assessments, indicating that diazepam did not decrease NAcc DA during cocaine self-administration. Findings that diazepam enhances cocaine self-administration and decreases cocaine response latency support the notion that cocaine-induced anxiety limits voluntary cocaine intake. It is further suggested that individual variations in cocaine-induced aversive effects may determine whether cocaine use is avoided or repeated.

[1]Reprinted from Pharmacol Biochem Behav, 91(1), Maier EY, Ledesma RT, Seiwell AP, Duvauchelle CL, Diazepam alters cocaine self-administration, but not cocaine-stimulated locomotion or nucleus accumbens dopamine, 202-7, Copyright (2008), with permission from Elsevier.

INTRODUCTION

Dopamine (DA) neural activation is associated with pleasurable effects of natural and drug-induced reward (Carelli 2004; Kelley and Berridge 2002) as well as aversive emotional states, such as fear and stress (Adinoff 2004; Pezze and Feldon 2004; Pruessner et al. 2004). This dichotomy may be epitomized during cocaine use, as the motivational effects of cocaine seeking are highly correlated with elevated nucleus accumbens (NAcc) terminal DA (Kiyatkin et al. 2000; Phillips et al. 2003), yet positive effects are often accompanied by an opposing state of anxiety. Indeed, human users have commonly reported that anxiety and panic attacks occurred after initial pleasurable feelings elicited by cocaine (Bystritsky et al. 1991; Cox et al. 1990; Geracioti and Post 1991; Gunnarsdottir et al. 2000; Walfish et al. 1990). Cocaine-induced anxiogenic effects are also demonstrated with animal behavioral models, where certain conditions of cocaine administration result in approach/avoidance and escape, as well as defensive postures and activities, such as crouching and directed sniffing (Blanchard and Blanchard 1999; Blanchard et al. 1998; DeVries and Pert 1998; Ettenberg 2004; Ettenberg and Geist 1991; Paine et al. 2002).

Anxiety and fear denote expectations of danger (Delgado et al. 2006) lead to danger avoidance, as a means for increasing survival. In regards to cocaine use, self-protective feelings, including cocaine-induced anxiety and panic, may be defense mechanisms promoting anti-addictive behavior (David et al. 2001). Therefore, an individual's emotional response to cocaine may determine whether initial cocaine use is followed by repeated, increased or termination of drug-taking behavior. If anxiety works to decrease cocaine intake, it is therefore conceivable that treatments blocking anxiogenic

cocaine effects may lead to repetitive cocaine use, and serve as "pro-addictive" agents that could facilitate the transition from recreational user to cocaine abuser.

Benzodiazepines, a class of drugs with sedative-hypnotic, muscle-relaxant, anxiolytic and anticonvulsant properties (Charney et al. 2001) have been used for the treatment of cocaine-induced toxicity and seizures (Smith and Landry 1990; Spivey and Euerle 1990). Benzodiazepines can also reduce cocaine-induced anxiogenic behaviors in animals (David et al. 2001; Ettenberg and Geist 1991; Paine et al. 2002) and are known to be co-administered with cocaine in human users (Wolf et al. 2005). Though benzodiazepine use alone is rarely lethal, post-mortem evidence indicates that benzodiazepine and cocaine co-administration can result in increased cocaine intake, toxicity and mortality (Wolf et al. 2005).

The present study was conducted to determine the influence of the benzodiazepine, diazepam, on cocaine self-administration and cocaine-induced DA enhancement in the NAcc. In Experiment 1, cocaine self-administration rates, latency to self-administer the first cocaine injection of the session and behavioral activity were assessed across twenty (20) daily cocaine self-administration sessions. Experiment 2 utilized in vivo microdialysis procedures to determine whether diazepam affects basal or cocaine-stimulated NAcc DA levels.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats, weighing approximately 250 g at the start of each experiment were used. *Ad libitum* access to food and water was provided, except during acquisition of food-reinforced operant training. The temperature in the colony was

maintained at 20° C and animals were kept on a 12-hour reverse light/dark cycle (lights off at 9 am). Rats were handled for 10 min daily for 2 weeks prior to the start of operant training. The experimental protocol was approved by the University of Texas Institutional Animal Care and Use Committee (IACUC) in compliance with NIH standards.

Apparatus

Food training, self-administration and in vivo microdialysis sessions for Experiments 1 and 2 were conducted in identical one-lever operant chambers (28 x 22 x 21 cm) within sound-attenuating boxes (Med Assoc, St. Albans, VT). The Plexiglas chambers contained a single retractable lever on the right wall, with a stimulus light directly above the lever and a house light on the opposite wall. Houselights were illuminated to signal the start of the sessions and availability of reinforcement. The stimulus light above the lever was activated with each lever response. Three pairs of photobeams monitored locomotor activity: one in the center and two others, each located 5 cm from the chamber walls. Interruptions between photobeam pairs were assessed as units of locomotor activity. Cocaine injections were delivered via 10 ml syringe positioned in a pump that was connected by tubing to a single-channel swivel mounted on a counterbalanced arm above each chamber. The tubing was connected to the animal using a spring-covered tether that screwed onto the jugular catheter endpiece located on the animal's head (Plastics One, Roanoke, VA). Operant programs and data collection was controlled by a MED P4 Intel computer system using MED-PC Software (Med Assoc, St. Albans, VT).

Food Training

Following a handling period of two weeks, all animals were food restricted and trained in the operant chambers to lever press for sucrose pellets (45 mg, P. J. Noyes, Lancaster, NH) on a fixed ratio-1 (FR-1) schedule of reinforcement. After the lever press response was acquired, 10-min daily operant training sessions were continued for a minimum of 6 days without food restriction.

Surgical Procedures

After completion of operant training, all animals were implanted with a jugular catheter. Animals in Experiment 2 were also implanted with an intracranial guide cannula aimed above the NAcc to accommodate the insertion of a microdialysis probe one day before the test day. Rats were anesthetized with pentobarbital sodium (Nembutal®; 50 mg/kg, i.p.). Atropine sulfate (80 μg/rat, s.c.) was given prophylactically to prevent respiratory secretions. Supplemental chloral hydrate (100 mg/kg, i.p.) was given, if necessary, to prolong anesthesia. A Silastic catheter (0.625 mm o.d.) was inserted into the right jugular vein and advanced into the right atrium. The distal end of the catheter was connected to a cannula endpiece (Plastics One, Roanoke, VA), which was routed subcutaneously along the side of the neck and out an incision on the head. In Experiment 2, rats were implanted with unilateral cannula (22 g; Plastics One, Roanoke, VA) using the following stereotaxic coordinates for the NAcc in relation to bregma (flat skull position): AP: +1.7 mm; ML: ±1.7 mm; DV: _2.5 mm. These NAcc coordinates were chosen from experiments previously conducted in our laboratory (D'Souza and Duvauchelle 2006). The cannula end of the jugular catheter and the NAcc cannula were both affixed to the skull with dental acrylic. Stainless steel obturators were placed into the NAcc cannulae and moved daily to prevent obstruction. Gentamicin sulfate (50

mg/ml) was topically applied into the jugular incision to prevent infection. Rimadyl (5 mg/kg, s.c.) was given as a post-surgical analgesic. On days 1–7 post-surgery, jugular catheters were flushed daily with 0.1 ml of a saline solution containing 1U/ml heparin, 1000 U/ml streptokinase (Streptase®), and 67 mg/ml of the antibiotic Timentin. After day 7, catheters were flushed daily with the same solution minus the Timentin component. Animals were allowed to recover for one week prior to cocaine self-administration sessions.

Drugs and Groups

Diazepam (0.25 mg/kg; Abbot Laboratories, North Chicago, IL) was delivered through the intravenous catheter in volumes ranging from 14 – 19 μl followed by a 0.1 ml heparin/saline flush. Cocaine HCl (0.75 mg/kg/inj; RTI International, Triangle Park, NC) was diluted in sterile 0.9 % sodium chloride. Concentrations were adjusted according to individual weights so that each self-administered cocaine injection was delivered in a volume of 0.1 ml. Experiments 1 and 2 each consisted of two drug treatment groups: 1) Vehicle + Cocaine, and 2) Diazepam + Cocaine. These groups were treated with either saline (0.1 ml, i.v.) or diazepam (0.25 mg/kg, i.v.) prior to cocaine administration. Pilot work in our lab found that this particular diazepam dose reduced rat abdominal muscle tension, but did not visibly affect baseline locomotor activity.

Experiment 1: Cocaine Self-Administration

One week after surgery, animals in Experiment 1 underwent daily self-administration sessions. Animals were taken from their home cage, given their assigned pretreatment and placed back into the home cage for 30 min. Animals were then placed

into the operant chamber and the session commenced immediately with the illumination of the houselight. Cocaine self-administration was maintained on a FR-1 schedule of reinforcement, with each response resulting in a 0.75 mg/kg cocaine infusion through the intravenous catheter. Each cocaine injection was infused over 6 sec, during which time the stimulus lamp was illuminated. After each infusion, there was a 20-sec "time-out" period, during which time the lever was retracted and no infusions could be delivered. Self-administration sessions were one hour in duration with a limit of 29 responses per session. In cases that maximum lever responses were obtained prior to the end of the 1-hr session, the house light turned off and the animal was removed from the chamber. After each session, the indwelling jugular catheters were flushed with 0.05 ml solution of streptokinase/heparin (see Surgery). Self-administration sessions were conducted between 10 am and 2 pm daily, 5 days/week, for a total time of 20 days (4 weeks). For Experiment 1, the number of lever responses, response latency (for first cocaine injection), and locomotor activity measures (photobeam breakages) were assessed daily and recorded by MED-PC software.

Experiment 2: Cocaine-stimulated Extracellular NAcc DA

In Vitro Recovery Calibration

Microdialysis probes were constructed as previously described (Duvauchelle et al. 2000), with an active membrane length of 2.5 mm at the probe tip. Prior to probe recovery, all probes were flushed with nanopure water. On the day of probe calibration, 1.0 ml gastight Hamilton 1000 series syringes were filled with freshly prepared filtered Ringer's solution (128.3 mM NaCl, 1.35 mM CaCl2, 2.68 mM KCl, and 2.0 mM MgCl2), and pumped through the probe at 1.6 μl/min. The probe tips were kept in a

beaker with Ringer's solution containing ascorbic acid (1.0%) and 5 nM DA, maintained at 37° C. Two 10-min samples from each probe were collected and assayed by high performance liquid chromatography with electrochemical detection (HPLC-EC). Probe recoveries were calculated by comparing the average peak heights of the two probe samples to those from a 25% dilution standard (1.25 nM DA). The mean (± SEM) recovery of probes used in the experiment was 14.75 (± 0.51).

Microdialysis Probe Implatation

After a 1-week recovery from jugular catheter/stereotaxic surgery, cocaine-naïve animals in Experiment 2 were briefly anesthetized with 1.5% isoflurane and implanted with a microdialysis probe through the previously implanted NAcc guide cannula. Each microdialysis probe was connected to a 1.0 ml gastight Hamilton 1000 series syringe mounted on a syringe pump (Razel®, Model A), and freshly prepared Ringer's solution (pH = 7.3) was pumped through the probe. Animals implanted with the probe remained in a holding chamber overnight with the syringe pump speed set at 0.2 μl/min. Bedding, food, and water were available in the holding chamber. Two hours prior to the test session, the pump speed was increased to 1.6 μl/min.

NAcc DA Test Session

For the in vivo microdialysis experiment (Experiment 2), microdialysis samples were collected at 10-min intervals for the duration of the 120-min test session. The session progressed as follows: Animals were placed in the operant chamber and three baseline dialysate samples (30-min total) were collected. Animals then received their assigned pretreatment (diazepam or saline through the i.v. catheters), followed by the

collection of three pretreatment dialysate samples (30-min total). The final hour of the session consisted of programmed intravenous injections of cocaine (0.75 mg/kg, i.v. x 2; time 0 and 10), with dialysate samples continuously collected.

Assay of Dialysis and Recovery Samples

The dialysate and recovery samples were analyzed for DA using HPLC-EC equipped with Shizeido capcell C-18 narrow bore column, ESA Model 5200 A Coulochem II Detector, a Model 5020 Guard Cell and a Model 5041 amperometric analytical cell. The mobile phase contained 150 mM Na_2HPO_4, 50 μM EDTA, 4.5 mM ~ 6.0 mM sodium dodecyl sulfate, 4.76 mM citric acid, 12.5 % (v/v) acetonitrile, 12.5 % (v/v) methanol (pH = 5.6). The analytical cell potential was set at + 200 mV (oxidation). The detection limit for DA was calculated at 0.05 pg with a signal/noise ratio of 3:1. Flow rate was set at 0.2 ml/min and 10 μl samples were manually injected. The amount of DA within each dialysate sample was determined by comparison with standards (DA HCl, Sigma, St. Louis) prepared and analyzed on the day of sample analysis. Data were collected and analyzed using an ESA Model 500 Data station.

Histological Analysis

Animals in the dialysis experiment were euthanized by an overdose of pentobarbital sodium and perfused with isotonic saline and 10% formalin. Brains were removed and stored in 10% formaldehyde/30% sucrose solution. The probe placements within NAcc (see Fig. 1) were verified from coronal sections (60 μm) stained with cresyl violet using the atlas of Paxinos and Watson (Paxinos and Watson 1998).

Statistic Analyses

For Experiment 1, the number of lever responses, locomotor activity and cocaine response latency (e.g., elapsed time between lever availability and first self-administered cocaine injection) across daily cocaine self-administration sessions in the diazepam- and saline- pretreated groups were compared using two-way repeated measures ANOVAs. Mean totals (sessions 2–20) of cocaine response latencies were also compared between groups using a two-sample t-test (two-tailed probability). For Experiment 2, extracellular NAcc DA concentrations (nM concentrations corrected according to probe recovery values) were analyzed using a two-way ANOVA with repeated measures at each 10-min interval across the 120-min test session. Since two-way interaction effects were not observed in any of the above ANOVA comparisons, posthoc tests were not performed.

RESULTS

Experiment 1: Lever Responses

A two-way ANOVA performed on number of lever responses across the 20 daily cocaine self-administration sessions showed significant Group [$F(1,19) = 8.0368$; $p = 0.01$] and Time effects [$F(19,361) = 10.8$; $p < 0.0001$], but no significant Group x Time Interaction [$F(19,361) = 0.64$; n.s.] (see Fig. 2).

Experiment 1: Locomotor Activity

A two-way repeated measures ANOVA across the 20 self-administration sessions showed no significant Group [$F(1,19) = 1.150$; n.s.] or Interaction effects [$F(19,361) = 1.069$; n.s.], but significant effects of Time [$F(19,361) = 3.75$; $p < 0.0001$] (see Fig. 3).

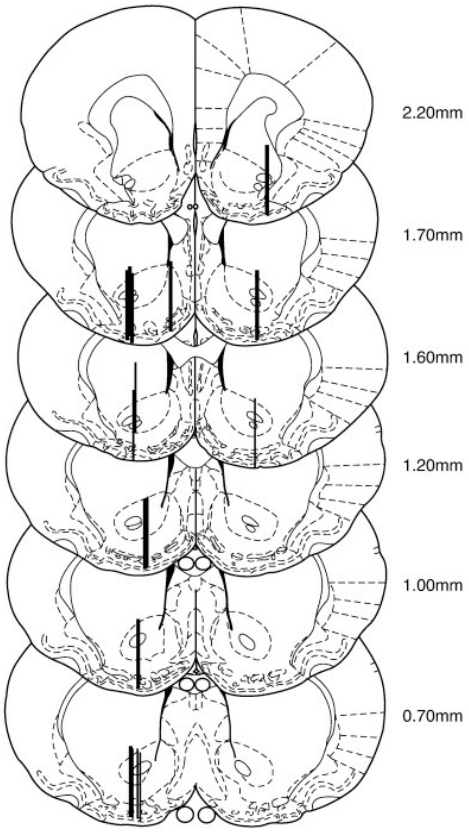
Experiment 1: Response Latency

This measure was defined as the time interval between the start of each self-administration session (e.g., houselight on and lever available) and the first lever press for cocaine. Latencies were assessed from sessions 2–20, as animals were cocaine-naïve prior to session 1. A two-way repeated measures ANOVA across sessions 2–20 showed significant Group effects [$F(1,19) = 5.68$; $p = 0.02$], but no significant effects of Time or Group x Time Interaction [$F(18,342) = 1.14$ and $F(18, 342) = 0.78$, respectively, both n.s.]. A two-sample t-test for independent groups revealed that the mean response latency for diazepam-pretreated animals was significantly lower than the vehicle-pretreated group [$t(19) = 2.18$; $p = 0.042$] (see Fig. 4).

Experiment 2: NAcc DA Levels

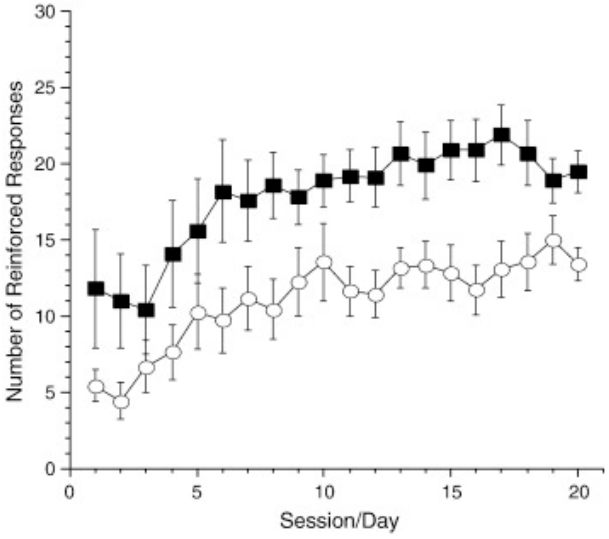
A two-way ANOVA with repeated measures on the 10-min intervals of the test session showed no significant Group [$F(1,14) = 2.37$; n.s] or Group x Time Interaction [$F(11,154) = 1.53$; n.s.], but significant effects of Time [$F(11,154) = 68.89$; $p < 0.0001$] (see Fig. 5).

Figure 1: Locations of Dialysis Probes



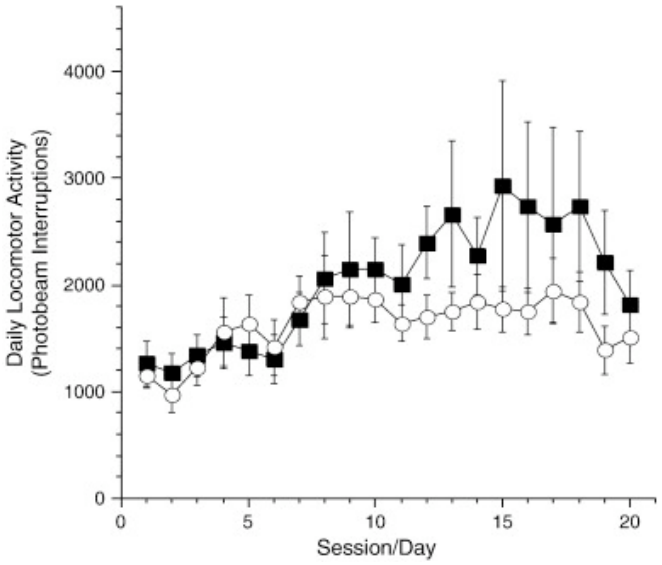
Locations of dialysis probe membranes within the NAcc (n = 16)/Experiment 2. Thickest lines represent probe locations for diazepam-pretreated animals.

Figure 2: Lever Responses



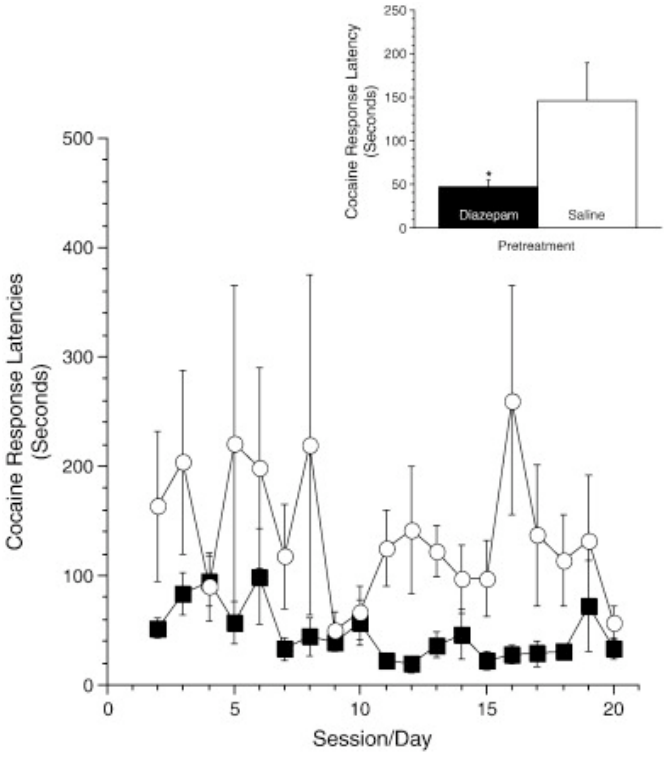
Daily lever responses during cocaine self-administration sessions/Experiment 1. Data represents mean ± SEM of cocaine-reinforced responses (0.75 mg/kg/inj) in diazepam- (filled square; n = 10) and saline- (open circle; n = 11) pretreated animals.

Figure 3: Locomotor Activity



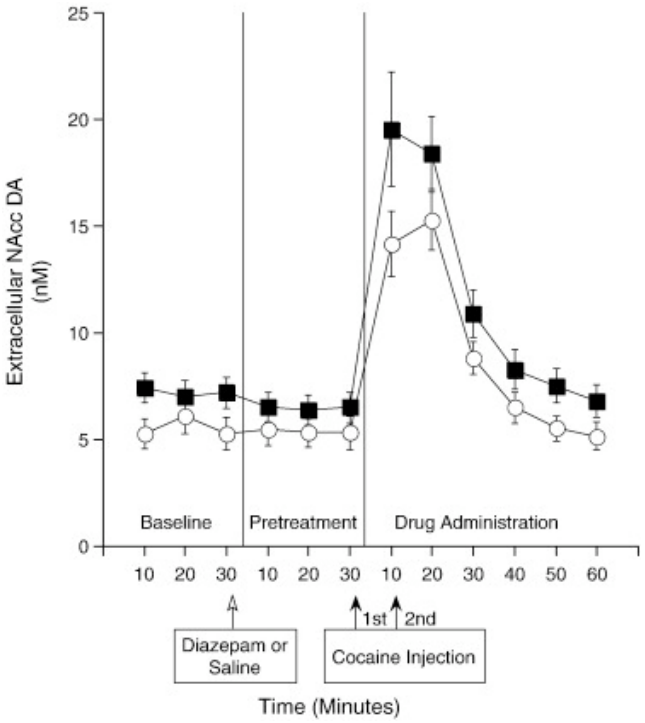
Locomotor activity during cocaine self-administration sessions/Experiment 1. Data represents mean ± SEM of locomotor activity (recorded as number of photobeam breakages within operant chamber) in diazepam- (filled square; n = 10) and saline- (open circle; n = 11) pretreated animals. No significant group differences in locomotor activity were observed across sessions.

Figure 4: Response Latency



Cocaine response latency/Experiment 1. Data represents mean ± SEM of elapsed time prior to the first operant response of cocaine self-administration sessions 2–20 in diazepam- (filled square; n = 10) and saline- (open circle; n = 11) pretreated animals. Insert: data depicted as mean ± SEM of sessions 2–20. Diazepam pretreated rats responded significantly faster for the first cocaine injection of each session than control animals. Significant difference at $p < 0.05$.

Figure 5: NAcc DA Response



NAcc DA response to experimenter-administered cocaine injections/Experiment 2. DA expressed in nM concentrations (mean ± SEM). No significant group differences in NAcc DA levels were detected for diazepam (filled square; n = 9) and saline (open circle; n = 7) pretreated animals.

DISCUSSION

Diazepam alleviates cocaine-induced anxiogenic-like behavior in animals (Blanchard and Blanchard 1999; Blanchard et al. 1998; DeVries and Pert 1998; Ettenberg and Geist 1991; Tarr and Macklin 1987; Wesson and Smith 1985). In Experiment 1, diazepam-pretreated animals made significantly more lever responses and responded significantly faster to obtain their first cocaine injection of each session compared to control animals. As suggested previously (Ettenberg and Geist 1991; Geist and Ettenberg 1997), diazepam treatment may shift the mixed emotional effects of cocaine to the positive side by alleviating negative, anxiogenic effects. Indeed, differences in response rates have often been attributed to changes in reward value and/or reinforcement efficacy (Caine et al. 1995; Hubner and Moreton 1991; Olmstead et al. 2000). However, based on past findings, increased levels of cocaine-maintained responding, as observed in diazepam-pretreated rats in the present study, may be interpreted as reflecting a decrease (Caine et al. 1995) or increase (Goeders 1997) in cocaine reinforcement efficacy. Though it has been argued that reinforcement efficacy cannot be determined through self-administration rates alone (Arnold and Roberts 1997), if diazepam acting to decrease negative effects of cocaine, the mechanisms for those effects may be distinct from those acting on the positive reinforcing aspects of cocaine. For instance, the tendency of control animals to hesitate longer than diazepam-treated animals in responding for their first cocaine injection during self-administration sessions suggests cocaine-induced anxiety acts to increase response latency. Therefore, as diazepam is proposed to attenuate the anxiogenic properties of cocaine inhibiting self-administration, diazepam treatment makes it possible for a greater number of lever responses to be completed during a session. Diazepam has also been shown to increase responding for punishment-

suppressed food (Johnson 1978; Thiebot et al. 1979) and brain stimulation (Moriyama et al. 1984) and to decrease anxiety associated with open fields and novel objects (Hoplight et al. 2005). Though we observed marked variability in response latency for vehicle-pretreated rats self-administering cocaine (as depicted in Fig 4), taken together, past and present data imply that diazepam enhances behaviors suppressed by a variety of anxiogenic factors.

The present study also observed that both the diazepam- and the saline-pretreated groups progressively increased lever responses for cocaine across sessions. These data concur with previous findings of enhanced cocaine responding during long-term self-administration (Emmett-Oglesby et al. 1993; Liu et al. 2005). Profound escalation in cocaine responding can also be observed over fewer sessions with longer daily access than provided in the current experiment (Ben-Shahar et al. 2004).

Diazepam is therapeutically used as a sedative/hypnotic agent and is thereby associated with attenuation in motion and performance. Some findings show acute use of diazepam is associated with decreased locomotor activity, and chronic use leads to increased locomotion (Djeridane et al. 2005) or behavioral tolerance to these effects (Fernandes et al. 1996; Mediratta et al. 2001). Others have found a range of effects on locomotor activity, including enhancement at lower doses of diazepam (0.25 mg/kg) (Soderpalm et al. 1991), no effects (Kiyatkin and Bae 2008) and attenuation at higher doses (0.5 mg/kg) (Soderpalm et al. 1991). However, in the present study, locomotor activity increased progressively in both groups, with no significant differences between control and diazepam-pretreated rats.

The diazepam-pretreated group of Experiment 1 showed differences in cocaine intake during self-administration sessions. Therefore, Experiment 2 was conducted to determine whether diazepam influenced extracellular NAcc DA. Diazepam has been

shown to affect ventral tegmental area DA neuronal firing rates and has dose-dependent decreasing effects on basal (Invernizzi et al. 1991) and cocaine-stimulated DA levels in the NAcc shell (Giorgetti et al. 1998). Therefore, it could be argued that, rather than decreasing cocaine-induced anxiety, the enhancement of lever responses observed in the diazepam group might reflect a compensatory behavioral response aimed at increasing DA levels to an "optimal" range achieved by the control animals. However, our data indicate this was not the case, since we found that cocaine-stimulated extracellular NAcc DA levels in the diazepam-pretreated group were not lower than controls. Our findings that basal NAcc DA is not affected by a low dose of diazepam has been previously reported (Invernizzi et al. 1991). Yet, previous work to date showing inhibitory effects of GABA agonists on VTA DA neuronal activity (Giorgetti et al. 1998; Liu et al. 2005) would logically support predictions that NAcc DA, behavioral activity and abuse liability would all be decreased in subjects treated with diazepam in combination with cocaine. In the present study, our findings do not support such predictions. However, since the combined diazepam and cocaine dosages utilized in the current work have not been examined previously, the effects on behaviors and NAcc DA reported here are novel contributions to the literature.

It should be noted that although differences in the magnitude of DA response between self-administered and experimenter-administered drugs have been reported (Hemby et al. 1995), there are no reports indicating opposing effects of cocaine on DA responses between the different modes of intake. Therefore, Experiment 2 provides reasonable evidence that cocaine-stimulated NAcc DA responses were enhanced for both groups during Experiment 1, thus comparably influencing operant behavior. Though not empirically addressed in the present experiment, it is possible that other neurotransmitters and neuromodulators involved in stress, such as corticotropin releasing hormone (CRH),

adrenocorticotropic hormone (ACTH), norepinephrine (NE) and serotonin (5-HT) which interact with GABAergic neurotransmission, may have contributed to the differences in behaviors observed between the two treatment groups (Carrasco and Van de Kar 2003).

Findings from the present study suggest that anxiety alters cocaine self-administration behavior in a manner that is responsive to enhanced GABA activation. Though the negative effects of cocaine are less examined than the positive rewarding effects, these findings suggest that cocaine intake may be increased in individuals co-administering sedative drugs. Correspondingly, therapeutic interventions that maximize the aversive effects of cocaine may be a novel means of decreasing cocaine use and abuse.

Chapter 3: Repeated Intravenous Cocaine Experience: Development and Escalation of Pre-Drug Anticipatory 50-kHz Ultrasonic Vocalizations in Rats [2]

ABSTRACT

Ultrasonic vocalization (USV) in the 50-kHz range occurs in rats immediately upon first-time exposure to cocaine or amphetamine, and rapidly increases with repetitive drug exposure at the same dose. This sensitized positive-affect response to these drugs of abuse is persistent in that the peak level of USVs again appears when the drug is reintroduced after several weeks of drug discontinuation. The present study explored whether with enough experience USVs might be elicited, and gradually escalate, in anticipation of impending drug delivery. Rats were trained to self-administer (SA) cocaine intravenously by lever pressing 5 days per week for 4 weeks. Yoked ratsreceived experimenter-delivered cocaine matching that of SA rats. USVs and locomotor activity were recorded during each 10-min period prior to 60-min drug access sessions. Extinction trials in which drug access was denied were then carried out over an additional 4-week period. After about a week of cocaine experience, both the SA and yoked groups began to progressively increase USVs when placed in an environment that predicted forthcoming drug exposure. Extinction of anticipatory calls and locomotion occurred over days after drug access ended. USVs may be a useful model for specifically investigating the neural basis of drug anticipation and aid in developing and assessing new addiction treatment strategies for reducing craving and relapse.

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[2]Reprinted from Behav Brain Res, 212(1), Ma ST, Maier EY, Ahrens AM, Schallert T, Duvauchelle CL, Repeated intravenous cocaine experience: development and escalation of pre-drug anticipatory 50-kHz ultrasonic vocalizations in rats, 109-14, Copyright (2010), with permission from Elsevier.

INTRODUCTION

Drug dependence is characterized by a pathological obsession to consume a drug. In susceptible individuals, predisposing vulnerability factors that facilitate stimulus-reward associative learning may influence the progression from controlled social drug use to compulsive drug abuse (Kalivas and O'Brien 2008; Robinson and Kolb 2004). Pairing environmental stimuli with stimulant drugs can lead to marked strengthening of drug-cue associations (Robinson and Berridge 1993; Robinson and Berridge 2008). With extended experience, exposure to drug-paired cues alone can increase activity in midbrain and forebrain dopamine systems, enhance locomotion (Duvauchelle et al. 2000; Weiss et al. 2000) and promote drug self-administration (Panlilio et al. 1996; Weiss et al. 2001). In addition, drug cues can acquire incentive-motivational value (Stewart et al. 1984). For example, cues previously associated with cocaine can become conditioned reinforcers, meaning that rats will lever press for presentation of the cue alone even when it is no longer accompanied by the drug (Di Ciano et al. 2007; Everitt and Robbins 2005). Learned drug-cue associations elicit strong ratings of craving inhuman cocaine dependents and have been reported to trigger relapse during abstinence (Wallace 1989). This suggests that the formation of drug-cue associations has a high impact on the development, progression and treatment of cocaine dependence.

In humans, drug craving and/or anticipatory attention very often occur, and increase, even in the absence of overt drug seeking behaviors (e.g., when trying to quit). Their occurrence and intensity can be estimated by verbal or checklist responses, but have been difficult to model adequately in animals. In rodents, increases in locomotor activity, rotational behavior (in the unilateral DA depletion model) and repetitive stereotyped movements (collectively termed “psychomotor” activity) have been proposed

by many to reflect drug appetitive behavior and motivation (Anagnostaras et al. 2002; Ferrario et al. 2005; Flagel and Robinson 2007; Pierce and Kalivas 2007). However, relationships between behavioral activity and motivational processes are indirect at best. Yet, it is possible that a class of ultrasonic vocalizations (USVs) may be an additional and very useful marker of anticipation of drug availability, or even craving.

Rats vocalize in ultrasonic frequencies that serve a social and communicatory function and express subjective emotional states (Brudzynski 2009; Knutson et al. 2002). Long calls (> 0.3 s) in the 22-kHz range are thought to reflect negative emotional states associated with anxiety and distress, since these calls are elicited by aversive stimuli such as predatory odors, footshock cues, social defeat, and aversive drugs (Knutson et al. 2002; Wohr and Schwarting 2008). Conversely, short calls (< 0.3 s) in the 50-kHz range are thought to reflect a state of positive affect associated with appetitive behavior, since these calls are increased during or in anticipation of mating, social play, or food presentation and are associated with reinforcement-related dopamine activity (Bialy et al. 2000; Burgdorf and Panksepp 2006; Knutson et al. 1998; Knutson et al. 2002). For example, very large increases in 50-kHz range calling have been elicited by electrical brain stimulation in areas that support self-stimulation (Burgdorf et al. 2007) and by systemic injections of cocaine (Mu et al. 2009) or d-amphetamine (Ahrens et al. 2009). Caffeine, at doses that increase locomotion, does not evoke USVs, in contrast to amphetamine, which does (Simola et al. 2009). 50-kHz USVs elicited by natural or pharmacological reward can be blocked or acoustically degraded by the loss of midbrain dopamine neurons or pretreatment with dopamine receptor antagonists (Burgdorf et al. 2007; Ciucci et al. 2009; Ciucci et al. 2007). Increases in the emission of 50-kHz calls have been linked to environments previously paired with drugs of abuse. In conditioned place preference (CPP) studies rats emit more 50-kHz calls in the drug-paired side of a

CPP chamber than in the vehicle-paired side (Knutson et al. 1999), and the number of 50-kHz calls elicited by an acute intracranial opioid injection predicts the subsequent development of CPP for the drug-paired side (Burgdorf et al. 2007). In addition, not only are 50-kHz calls in male rats emitted when odor cues of sexually receptive females are presented, these calls increase dramatically with repeated mating experience (Bialy et al. 2000; Ciucci et al. 2007). Recent research indicates that 50-kHz calls occur immediately and very rapidly increase with repeated exposure to cocaine or amphetamine during the periods in which the drugs are on-board (Ahrens et al. 2009; Mu et al. 2009). However, the appearance and gradual escalation of USV production during pre-drug periods as the rats learn to expect drug availability has not yet been demonstrated. Indeed, investigators examining USVs across 5 days of cocaine sessions did not observe cocaine anticipatory USVs (Ahrens et al. 2009; Mu et al. 2009). We hypothesized that more trials might be needed for adequate conditioning to take place.

The primary goal of the present study was to examine whether, with repeated sessions of cocaine administration, 50-kHz calls will progressively increase in anticipation of impending cocaine delivery and, with extinction, decrease in anticipation of not receiving cocaine. USVs may become a useful adjunct model for specifically investigating neural changes associated with drug anticipation ("looking forward to" drug delivery). If so, this measure could become an important tool to design and confirm the effectiveness of treatment strategies aimed at reducing drug associations, wanting, craving and/or relapse.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were housed in an animal colony in polypropylene cages (45 cm x 23.75 cm x 20 cm) under a 12:12 reversed light/dark cycle (lights off at 8 am). Prior to lever response training, rats were handled for 10 min a day for a period of 2 weeks, with food and water provided *ad libitum*. Animals were approximately 9–10 weeks old at the start of cocaine conditioning sessions. Sessions were conducted daily between 10 am and 2 pm, 5 days a week.

Apparatus

Lever response training, cocaine conditioning and extinction sessions were conducted in one-lever operant chambers (28 cm x 22 cm x 21 cm) housed within sound-attenuating boxes (Med-Associates, St. Albans, VT) to limit outside noise and light. During the administration sessions, the animals were intravenously connected via tubing to a syringe mounted on a motorized pump (Razel Scientific Instruments, Model A, St. Albans, VT) containing either a cocaine solution (0.75 mg/kg/injection) or sterile saline. After each lever press, cocaine or saline was administered over a 6-s infusion time and a stimulus light above the lever blinked for the duration of the infusion. The operant chamber was also equipped with a house light and 3 sets of photocells; two located 5 cm from each sidewall and one in the middle of the chamber. The number of interruptions of the photobeams was assessed in 10-min intervals as a measure of locomotor activity. For USV recordings, an ultrasonic microphone (PCB Piezotronics, Buffalo, NY) with a flat

frequency response ranging from 10-kHz to 100-kHz, was securely mounted over the center of the operant chamber. Recordings were then digitally stored and analyzed later.

Self-Administration and Yoked Groups

After the handling phase, the animals were divided into self-administration (SA) and yoked (Y) groups. In the SA groups, rats were trained for lever response (see below) and later allowed to self-administer cocaine or saline. In the Y groups, rats were matched with a member of the SA group and passively received the same amount of treatment (food pellets, cocaine or saline infusions) as its self-administering counterpart.

Lever Response Training

Food-restricted animals in the SA group learned to lever press for sucrose pellets (45 mg) on a fixed ratio-1 (FR-1) schedule of reinforcement. All rats had 12 days of 10-min lever response training sessions on the FR-1 schedule in which each lever response resulted in illumination of a stimulus light (1 s) above the lever and delivery of a sucrose pellet. Food-restricted yoked animals were placed into the operant chamber under the same conditions, in the absence of the lever, and received the same daily schedule of sucrose pellet delivery as their lever-pressing counterparts. After the 12 days of lever response training, all animals underwent jugular catheterization surgery.

Jugular Catheterization Surgery

All animals were surgically implanted with a Silastic catheter under anesthesia composed of a mixture of oxygen (0.8 – l/min; Airgas Southwest, Corpus Christi, TX) and isoflurane (2.5 – 4 %; AErrane, Baxter Healthcare, Deerfield, IL) delivered through a

gas delivery system (VetEquip, Inc, Pleasanton, CA). Catheters were constructed from 8.5 cm Silastic tubing (0.64 mm o.d.) with one end connected to a cannula endpiece (Plastics One, Roanoke, VA). The catheter was inserted into the right jugular vein and the cannula endpiece was passed subcutaneously to an incision on the head. The catheter was anchored to the top of the head with four stainless steel screws and dental acrylic cement. Just prior to the closing suture, gentamicin sulfate (50 mg/ml) was applied (3 drops) into the jugular incision to prevent infection. The anti-inflammatory agent, carprofen (5 mg/kg, s.c.) was given as a prophylactic for post-surgical pain. Rats were allowed to recover from the surgery for one week prior to starting the cocaine conditioning sessions. During this time, catheters were flushed daily with 0.1 ml of a solution containing an antibiotic (Timentin; 100 mg/1.5 ml) dissolved in heparinized saline. Animals continued to receive the same solution daily without the antibiotic component through the duration of the experiment to maintain catheter patency.

Conditioning and Extinction Sessions

At the start of each conditioning session, the animals were placed into the chambers and connected to the drug delivery tubing. The chamber remained dark for a 10-min interval prior to the start of cocaine or saline availability. During this time, the lever was withdrawn from the chamber to ensure that no opportunities for lever responding were possible. At the end of this interval, a house light within the chamber illuminated, the lever extended into the chamber, and SA rats were able to perform lever presses for cocaine or saline over a period of 60 min. Each lever press resulted in a cocaine or saline infusion to themselves and to their yoked counterpart. Extinction sessions commenced the day after the 20th conditioning session (e.g., Day 21). During

extinction, the house light and lever were activated exactly as in conditioning sessions, but all lever responses by the SA rats resulted in saline infusions (0.1 ml) for themselves and their yoked counterparts. Conditioning and extinction sessions were conducted 5 days per week, for approximately 8 weeks (e.g., 20 conditioning and 19 extinction sessions).

Data Collection

USV and locomotor activity (photobeam interruptions) data were collected during each 10-min (dark) interval prior to cocaine availability or extinction sessions. Locomotor activity was recorded by MED-PC software (Med Associates, St. Albans, VT). USV data were captured by ultrasonic microphones (detection range = 10 - 100 kHz), recorded by RECORDER multi-channel recording software and analyzed by SASLab Pro analyzing software (Avisoft Bioacoustics, Berlin, Germany).

Drugs

Cocaine HCl (0.75 mg/kg/injection, RTI International, Triangle Park, NY) was diluted in sterile 0.9% sodium chloride daily and adjusted according to each individual rats' weight so that each cocaine-reinforced lever response resulted in a 0.1 ml delivery of cocaine solution at the 0.75 mg/kg dosage.

Statistical Analyses

USVs and locomotor activity were recorded during the 10-min periods prior to each conditioning and extinction session. 50-kHz USV data and locomotor activity were analyzed using two-way repeated measures ANOVA (e.g., Group _ Days), with "Days"

as a within-subjects variable and "Group" (SA cocaine, yoked cocaine, SA saline and yoked saline) as a between-subjects variable. Due to equipment or computer tabulation error, 9 out of 1,833 USV data points and 12 out of 1,833 locomotor activity intervals were lost. In these cases, group means were inserted so that collected data from each animal could be included in overall analyses. Significant interaction effects were further analyzed using post hoc tests (Fishers LSD) with significant comparisons reported as $p < 0.05$ or $p < 0.01$.

RESULTS

Acquisition

50-kHz USVs

There was a significant increase in the number of 50-kHz USVs elicited in the cocaine-paired context (during the pre-drug interval) over repeated days of drug exposure (see Fig. 1). Two-way repeated measures ANOVA comparing all treatment groups during the interval prior to cocaine availability showed significant Group [$F(3,21) = 3.92$; $p = 0.023$], Day [$F(19,399) = 5.296$; $p < 0.001$] and marginal Group x Day interaction effects [$F(57,399) = 1.337$; $p = 0.060$]. Post hoc analyses revealed that both cocaine-receiving groups (SA and yoked cocaine) elicited significantly more USVs prior to cocaine conditioning sessions than both control groups (SA and yoked saline) prior to saline availability (see Fig. 6). Sample USVs sonograms and slowed-down calls can be viewed and heard at the following web sites:

http://www.utexas.edu/pharmacy/divisions/pharmtox/faculty/duvauchelle2.html and www.schallertlab.org (Media).

Locomotor Activity

In the 10-min interval prior to cocaine availability, overall locomotor activity was higher in rats about to receivecocaine compared to Controls (see Fig. 7). Significant Group [$F(3,21) = 8.647$; $p = 0.001$] and Group x Day interaction [$F(57,399) = 1.727$; $p = 0.002$], but no significant Day effects [$F(19,399) = 1.147$; n.s.] were observed. Post hoc analyses revealed SA cocaine rats had the highest activity levels overall and were significantly greater than both saline groups ($p < 0.01$). Locomotor activity in the yoked cocaine group was significantly higher than the yoked saline group ($p < 0.01$) and (marginally) significantly greater compared to the SA saline group ($p = 0.065$; see Fig. 2).

Extinction

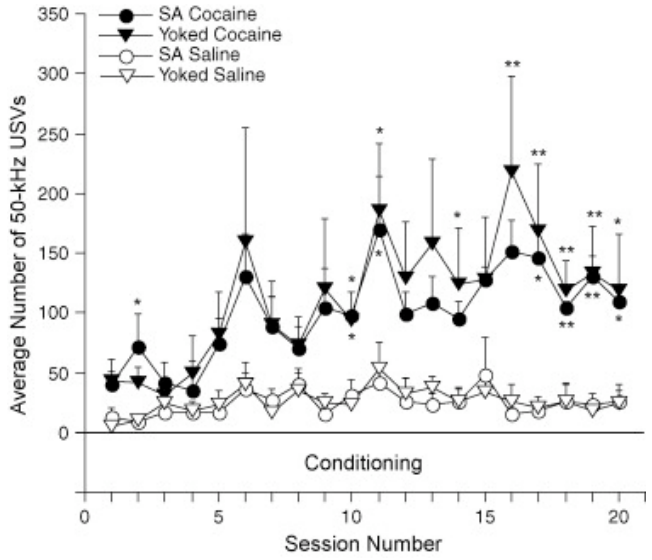
50-kHz USVs

During the 10-min intervals just prior to extinction sessions, the cocaine anticipatory USVs significantly decreased once cocaine was no longer available during extinction sessions (see Fig. 8). A two-way repeated measures ANOVA revealed significant Group [$F(3,19) = 4.54$; $p = 0.015$], Day [$F(18,342) = 5.69$; $p < 0.001$] and Group x Day interaction effects [$F(54, 342) = 1.71$; $p = 0.002$]. Post hoc analyses indicated that across extinction sessions, USVs were significantly greater in the yoked cocaine (but not SA cocaine) group than either saline group. Fig. 8 shows cocaine anticipatory USVs in the SA cocaine group dropping to control levels before USVs in the yoked cocaine group.

Locomotor Activity

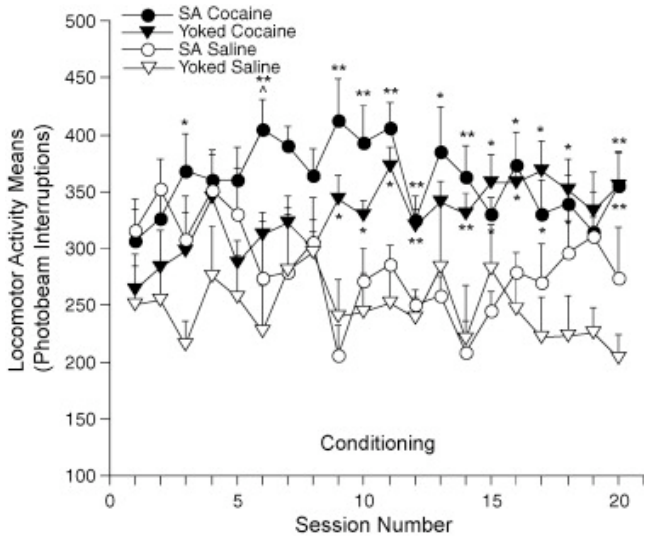
In the 10-min intervals prior to the extinction sessions, cocaine-conditioned locomotor activity in the SA cocaine group decreased across sessions but remained significantly greater overall than the yoked saline group ($p < 0.01$) (see Fig. 9). Two-way repeated measures ANOVA showed significant Day [$F(18,342) = 3.586$; $p < 0.001$), marginal Group [$F(3,19) = 2.954$; $p = 0.059$], but no significant Groups x Day interaction effects [$F(54,342) = 0.785$; n.s.].

Figure 6: Anticipatory USV Acquisition.



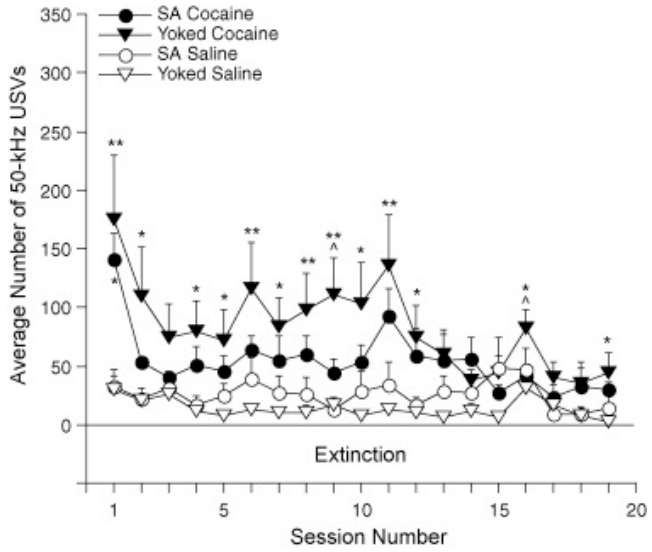
50-kHz USVs (mean ± SEM) of SA (self-administering) and yoked rats recorded during daily (Days 1–20) 10-min intervals prior to conditioning sessions for SA cocaine (filled circle, n = 7), yoked cocaine (filled trident, n = 6), SA saline (open circle, n = 6) and yoked saline (open trident, n = 5) groups. Mean USVs across all conditioning sessions were significantly greater in both cocaine groups compared to either saline group. Posthoc comparisons (LSD) revealed differences between groups on specific days. *, ** = significantly more USVs elicited than either or both saline conditions; $p < 0.05$ or $p < 0.01$, respectively. No long-duration 22-kHz calls were detected in any animals during this interval.

Figure 7: Conditioned Locomotor Activity During Acquisition



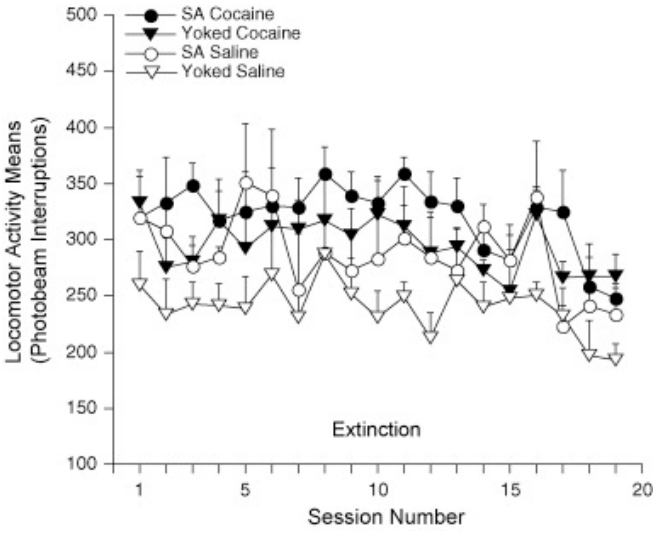
Locomotor activity (photobeam interruptions) recorded during 10-min intervals prior to conditioning sessions. Data presented as the mean (± SEM) daily intervals for SA cocaine (filled circle) yoked cocaine (filled trident), SA saline (open circle) and yoked saline (open trident) groups (same animals as in Fig. 6). Mean locomotor activity across all pre-conditioning intervals was significantly greater in the SA cocaine group than either saline group, and in the yoked cocaine group compared to the yoked saline group ($p < 0.01$). Posthoc comparisons (LSD) revealed group differences on specific days. *, ** = significantly greater daily mean than either or both saline conditions; $p < 0.05$ or $p < 0.01$, respectively. ^ = significantly greater than yoked cocaine group; $p < 0.05$.

Figure 8: Anticipatory USVs During Extinction



50-kHz USVs (mean ± SEM) of SA (self-administering) and yoked rats recorded during daily (Days 21–39) 10-min intervals prior to extinction sessions for SA cocaine (filled circle, n = 6) yoked cocaine (filled trident, n = 6), SA saline (open circle, n = 6) and yoked saline (open trident, n = 5) groups. Mean USVs across extinction sessions were significantly greater in the yoked cocaine (but not SA cocaine) group than either saline group. Posthoc tests (LSD) revealed that USVs in the yoked cocaine group (but not the SA cocaine group) remained significantly higher than controls over several days. *, ** = significantly greater daily mean than either or both saline conditions; $p < 0.05$ or $p < 0.01$, respectively. No long-duration 22-kHz calls were detected in any animals prior to extinction sessions.

Figure 9: Conditioned Locomotor Activity During Extinction



Locomotor activity (photobeam interruptions) recorded during 10-min intervals prior to extinction sessions. Locomotor activity data presented as the mean (± SEM) daily intervals for SA cocaine (filled circle) yoked cocaine (filled trident), SA saline (open circle) and yoked saline (open trident) groups (same animals as in Fig. 8). Over the course of extinction sessions, locomotor activity in the SA cocaine group decreased, but was significantly greater overall compared to the yoked saline group ($p < 0.01$).

DISCUSSION

Daily cocaine experience led to a delayed and gradual increase in anticipatory 50-kHz vocalizations. After 4 weeks cocaine was discontinued, and over the course of extinction, USVs decreased as the animals learned to no longer expect drug delivery despite unchanging contextual cues. Together, the acquisition data can be reasonably attributed to the development of cue-related anticipation of cocaine availability, and the extinction data to dampening drug expectation.

Previous reports of USV sensitization with repeated exposure to amphetamine or cocaine were limited to the drug exposure periods (Ahrens et al. 2009; Mu et al. 2009). This is the first demonstration that these calls also increase progressively in response to situational cues (e.g., placement in the drug-delivery environment) that predict impending drug exposure. 50-kHz calls are associated with positive affect in a variety of non-drug situations (Burgdorf et al. 2008; Knutson et al. 2002). As noted previously, with sexual experience, 50-kHz calls increase dramatically in response to cues that predict the opportunity to mate (Bialy et al. 2000; Ciucci et al. 2009; Ciucci et al. 2007). These reports are consistent with the possibility that this class of calls may reflect learning-enhanced anticipation of, attention to, or perhaps craving for cocaine. Burgdorf and associates (Burgdorf et al. 2000) reported that rats that make more 50-kHz range calls in anticipation of 10-min of rewarding brain stimulation are more likely to engage in self-stimulation behavior than rats that do not emit anticipatory USVs. Thus, increases in the number of USVs may reflect enhanced incentive salience. Because drug craving/wanting is an important component in the development and persistence of drug dependence (Childress et al. 1999; Robinson and Berridge 1993), USVs may be a highly useful method for estimating the extent of drug wanting in animals (Panksepp et al. 2002).

Other work indicates that the mode of administration of drugs of abuse (e.g., self-administered versus yoked delivery) can have different influences on drug-induced behavior (Lecca et al. 2007) and neurochemical events in mesolimbic pathways of the brain (Hemby et al. 1997; Lecca et al. 2007; Miguens et al. 2008). In the present study, USV recordings revealed comparable escalation of 50-kHz USV in anticipation of cocaine administration between rats contingently or non-contingently receiving cocaine. Indeed, our findings suggest that the cocaine-receiving environment serves as a Pavlovian contextual cue for either 1) both the SA and yoked cocaine groups, or 2) just the yoked rats (Hayes and Gardner 2004), while the SA cocaine group associates the cocaine environment as an occasion-setter for operant responding (Holland 1991). In either case, since both groups showed anticipatory positive USVs in the cocaine context, it is clear that these USVs were not elicited only in anticipation of reinforced operant responding. However, since USVs in the 50-kHz range are associated with positive affect (Burgdorf and Panksepp 2006; Knutson et al. 2002), these findings may appear at odds with a recent study reporting that yoked cocaine delivery can be aversive and inhibit future cocaine-seeking behavior (Twining et al. 2009). Yet, it remains possible that drug anticipation/seeking may have underlying neural substrates that differ from that occurring during actual drug delivery. In the present study, no long-duration 22-kHz USV calls, which are thought to reflect aversive affect (Brudzynski 2009), were detected during the reported 10-min drug-free intervals. In any event, the progressive increase in anticipatory USVs is consistent with studies that have demonstrated long-lasting CPP with chronic psychostimulant exposure (Sakoori and Murphy 2005; Tzschentke 2007). Knutson and associates (Knutson et al. 1999) have argued that 50-kHz USVs may be more sensitive than CPP in estimating the degree of drug preference (especially for psychostimulants that are less potent than cocaine in the central nervous system). It is likely that CPP and

USVs, both of which may assess drug cue salience without the drug being on-board, together may provide more useful information than either alone. In addition, the reduction in USV calls during extinction supports the notion that this measure directly reflects both learned anticipation of forthcoming drug and unrewarded events. These data further suggest that USVs may be a reliable measure of the presence or absence of cocaine anticipation. It should be noted that during the extinction phase, the yoked cocaine group received non-contingent saline injections and did not show as rapid a decline in anticipatory USVs compared to the SA cocaine group. These findings suggest that USVs conditioned by yoked delivery of cocaine is resistant to extinction, or that yoked delivery of saline in a cocaine-associated environment does not suppress conditioned USVs as efficiently as does non-reinforced operant responding.

As expected, we found that locomotor activity during the 10-min periods prior to drug access was significantly greater in animals with cocaine experience. Others report enhanced behavioral activity in a cocaine-associated environment (Adams et al. 2000; Anagnostaras et al. 2002; Liu and Cunningham 2006). Cocaine-induced motor activity may be influenced, at least in part, by the activation of neural circuits known to support rewarding brain stimulation and drug reinforcement (Burgdorf et al. 2007), though it is unclear whether anticipatory locomotor activity is mediated by similar anatomical substrates (Sellings et al. 2006). In the present study, locomotor activity was electronically tabulated as the number of detected photobeam interruptions within the operant chamber, rather than by direct observation of behavior activity. Future research should address the possibility that qualitative analysis of cue-related motor activity may reveal, as with USV production, a more specific form of anticipatory behavioral activity that reliably increases incrementally with drug exposure.

Escalating motivation for and cognitive obsession about a drug over time are considered key features of addiction (Deroche-Gamonet et al. 2004). It is possible that with more extensive experience, 50-kHz USVs in rodents, along with sign-tracking (Flagel et al. 2008; Uslaner et al. 2008), CPP (Pelloux et al. 2009; Rodriguez-Arias et al. 2009) motivation to overcome obstacles or to work hard to gain access to a drug (Deroche-Gamonet et al. 2004; Vanderschuren and Everitt 2004) and related assessments (Stafford et al. 1998), may become a major indicator of the presence and extent of drug incentive motivation, hedonic state, drug anticipation and/or wanting. If so, then USVs may serve as pre-clinical model of craving that will help to develop treatments to block impulsive drug taking, relapse and obsessive attention to cues associated with drugs of abuse.

Chapter 4: Cocaine Deprivation Effect: Cue Abstinence over Weekends boosts Anticipatory 50-kHz Ultrasonic Vocalizations in Rats [3]

ABSTRACT

In drug dependence studies, rats are often tested daily with short breaks (such as weekends) spent untested in their home cages. Research on alcohol models has suggested that breaks from continuous testing can transiently enhance self-administration (termed the "alcohol deprivation effect"). The present study explored whether the salience of cocaine-access cues is increased after skipping weekend cocaine and cue exposures. Ultrasonic vocalizations (USVs) of the 50-kHz class are emitted by rats exposed to intravenous cocaine and have been shown to increase with repeated drug exposure at the same dose level (sensitization). The present study found that over the course of several weeks of cocaine self- or yoked-administration pre-drug cues signaling forthcoming access or delivery of cocaine elicited marked amounts of anticipatory 50-kHz USVs, and that weekend deprivation from cues and cocaine exaggerated further the level of calling (more calls on Mondays compared to Fridays). Anticipatory USVs extinguished less rapidly when weekend access to unreinforced cues was denied. The results may have clinical implications, in that intermittently avoiding cues or context may enhance drug cue salience and resistance to extinction.

[3]Reprinted from Behav Brain Res, 214(1), Maier EY, Ahrens AM, Ma ST, Schallert T, Duvauchelle CL, Cocaine deprivation effect: cue abstinence over weekends boosts anticipatory 50-kHz ultrasonic vocalizations in rats, 75-9, Copyright (2010), with permission from Elsevier.

INTRODUCTION

Cocaine is a highly addictive drug, especially in people vulnerable to overuse. Cues that reliably signal impending drug availability can lead to craving, particularly with extended stimulant drug-taking experience (Childress et al. 1999; Kalivas and O'Brien 2008; Kilgus and Pumariega 1994; Maas et al. 1998; Robinson and Berridge 1993; Robinson and Berridge 2008; Robinson and Kolb 2004) and can trigger relapse in abstinent cocaine addicts (Wallace 1989). In rats, cues that are paired with reinforcing drugs can increase synaptic dopamine and facilitate drug seeking (e.g., lever pressing) and psychomotor activity (Anagnostaras et al. 2002; Duvauchelle et al. 2000; Everitt and Robbins 2005; Flagel and Robinson 2007; Stewart et al. 1984). Furthermore, these effects have been shown to persist after cue extinction training (Di Ciano and Everitt 2002; Meil and See 1996; Panlilio et al. 1996; Weiss et al. 2000; Weiss et al. 2001). Unlike in people (Childress et al. 1999), assessing the degree of drug craving in the absence of drug seeking in animals has been challenging. However, accumulating evidence suggests that rat ultrasonic vocalization (USV) in the 35 – 80 kHz range, termed "50-kHz" USVs, may reflect ongoing positive hedonic affect as well as cue-related anticipation of upcoming highly reinforcing events. For example, sexually experienced male rats reliably emit 50-kHz USVs when exposed to female odors in the absence of the female, and these calls occur also in response to cues that signal other positive reinforcers such as food, social contacts and drugs of abuse (Bialy et al. 2000; Knutson et al. 1998; Knutson et al. 1999; Ma et al. 2010; Wohr et al. 2008). These studies suggest that USVs can be an indicator of a state of "looking forward to" impending reward and perhaps even an index for the level of drug expectation and wanting.

The present study confirmed that anticipatory 50-kHz USVs increase in rats repeatedly exposed to cues that predict upcoming intravenous cocaine access and delivery. In addition, this study showed that these calls increase further on the day following a 2-day period in which the rats were not exposed to the cues or the drug but instead remained in their home cages (deprivation of drug and cue/context).

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Charles River Laboratories, Inc., Wilmington, MA), 9 – 10 weeks old, were well-handled and housed in polypropylene cages under a reversed light/dark cycle (8 p.m. to 8 a.m.). Food and water were provided *ad libitum*. Animals were tested in the dark cycle, 5 days per week with weekends off.

Apparatus

One-lever operant chambers (28 cm x 22 cm x 21 cm) were used for lever response training, cocaine conditioning and extinction sessions. To limit external light and noise, these chambers were set in sound-attenuating boxes (Med-Associates, St. Albans, VT). For cocaine sessions, the rat's indwelling intravenous catheter was connected to tubing from a syringe mounted on a motorized pump (Razel Scientific Instruments, Model A, St. Albans, VT). The syringe contained either a solution of cocaine (0.75 mg/kg/injection) or sterile saline. The operant chamber was also equipped with a house light and a stimulus light above the lever. After each lever press, cocaine or saline was administered over a 6-s infusion time and the stimulus light above the lever illuminated for the duration of the infusion. An ultrasonic microphone was positioned

over the center of the chamber and USVs were digitally recorded and stored. Photocells were positioned 5 cm from each sidewall and in the middle of the chamber and photobeam interruptions were recorded in 10-min intervals to assess locomotor activity within the chamber in correspondence with USV recordings.

Self-Administration and Yoked Groups

Rats were divided into 4 groups: cocaine self-administration (SA, n = 8), cocaine yoked (Y, n = 6), saline SA (n = 6) and saline Y (n = 5). SA rats were first trained to press the lever for sucrose pellets and later allowed to self-deliver cocaine or saline. Y rats were matched for dose with one of the SA rats and passively received an identical amount of food pellets, cocaine or saline infusions as their self-administering counterpart.

Lever Response Training

SA rats were trained to lever press for sucrose pellets (45 mg, P.J. Noyes, Lancester, NH) on a fixed ratio-1 (FR-1) reinforcement schedule. 10-Min lever response training sessions occurred over 12 days. Lever presses illuminated a light above the lever, and was immediately followed by the delivery of a sucrose pellet. Yoked animals were treated identically except that no lever was made available.

Jugular Catheterization Surgery

Silastic catheters were implanted in all rats under anesthesia consisting of a mixture of oxygen (0.8 l/min; Airgas Southwest, Corpus Christi, TX) and isoflurane (2.5–4%; AErrane, Baxter Healthcare, Deerfield, IL) delivered via a gas delivery system (VetEquip, Inc., Pleasanton, CA). Catheters were made from 8.5 cm silastic tubing (0.64

mm o.d.) with one end connected to a cannula endpiece (Plastics One, Roanoke, VA). The catheter was inserted into the jugular vein on one side of the body. The endpiece of the cannula was subcutaneously passed to an incision on the head. Four stainless steel screws and dental acrylic cement were used to anchor the catheter to the dorsal surface of the head. To prevent infection, 3 drops of gentamicin sulfate (50 mg/ml) were applied to the jugular incision before closing the suture. Carprofen (5 mg/kg, s.c.), an anti-inflammatory agent, was also administered immediately after the surgical procedure. One-week recovery from surgery was allowed before starting conditioning sessions. To maintain catheter patency, the catheters were flushed daily with 0.1 ml heparinized saline (1 U/ml heparin). The antibiotic Timentin (100 mg/1.5 ml) was added to the flushing solution during the first week post-surgery.

Conditioning and Extinction Sessions

Chamber lights were turned off during the 10-min pre-drug anticipation intervals signaling impending cocaine or saline availability. Immediately following these intervals, the house light was illuminated and, at an unpredictable time point within a 30 s span, the lever was presented to SA rats, indicating that they were allowed to lever press for cocaine or saline over a period of 60 min. During this session, Y rats received unpredictable cocaine or saline injections according to the rate of lever pressing by their self-administering counterparts. Conditioning and extinction sessions were carried out 5 days per week, with no sessions occurring on weekends. After 4 weeks of conditioning, extinction sessions were conducted for 4 weeks, in which all lever presses yielded delivery of only saline (0.1 ml/infusion) to all groups.

Data Collection

USVs and locomotor activity were recorded every weekday (Monday – Friday) in operant chambers during the 10-min intervals prior to cocaine or saline availability or extinction sessions. Over the 2-day weekends, animals remained in the vivarium environment in their home cages. During experimental sessions, motor activity was recorded by MED-PC software (Med-Associates, St. Albans, VT) and USVs were recorded using ultrasonic microphones (PCB Piezotronics, Buffalo, NY; working frequency range: 5 – 126,000 Hz) mounted in the center of the operant chamber, inaccessible to the rat. The maximum distance of the microphone to any point within the chamber was approximately 26 cm. Therefore, as the rat was freely moving within the chamber at all times, the proximity between the microphone and rat varied during each session, but was always less than 26 cm. RECORDER multi-channel recording software and SASLab Pro software (Avisoft Bioacoustics, Berlin, Germany) were used to record and assess rat USVs. Sample 50-kHz USVs sonograms and slowed-down calls can be viewed and heard at the following web sites: www.schallertlab.org (Media section) and http://www.utexas.edu/pharmacy/divisions/pharmtox/faculty/duvauchelle2.html.

Drugs

Cocaine HCl (0.75 mg/kg/infusion) in sterile 0.9 % sodium chloride was adjusted daily according to body weight such that cocaine-reinforced lever responses led to delivery of 0.1 ml cocaine solution at the 0.75 mg/kg dose.

RESULTS

USVs and locomotor activity were recorded during the 10-min intervals prior to each conditioning and extinction session. Because the primary aim of the present study was to examine whether a 2-day (weekend) respite from exposure to the conditioning chamber affected the number of anticipatory USVs, we analyzed pre-drug session (cue only) data across all Fridays vs. Mondays during conditioning and extinction sessions. We also compared anticipatory USVs and locomotor activity data on Fridays with those on Tuesdays, Wednesdays and Thursdays and confirmed that each of these values did not significantly differ. Two-way ANOVAs showed no significant differences in 50-kHz USVs or locomotor activity levels between SA and Y groups during the pre-drug session intervals for either the cocaine or saline conditions. Therefore, cocaine-receiving groups were combined and the saline groups were combined (SA + Y cocaine and SA + Y saline) for analyses. Two-way ANOVAs compared effects of Treatment (cocaine vs. saline) with repeated measures on Days (Friday vs. Monday).

Conditioning Sessions

50-kHz USVs

Two-way repeated measures ANOVA showed significant Treatment ($F(1,23) = 11.49$; $p = 0.003$), Day ($F(1,23) = 9.327$; $p = 0.006$) and Treatment x Day interaction effects ($F(1,23) = 6.579$; $p = 0.017$). Post hoc tests revealed that USVs elicited during pre-drug intervals on both days (e.g., Fridays and Mondays) were significantly higher in the "cocaine-expected" condition compared to the "saline-expected" condition. In addition, the cocaine rats showed significant enhancement in the number of USVs on Mondays compared to Fridays ($p < 0.01$), but the saline rats did not (see Fig. 10).

Locomotor Activity

Two-way repeated measures ANOVA showed significant effects of Treatment ($F(1,23) = 20.38$; $p = 0.000$), but no significant differences were observed for Day ($F(1,23) = 2.418$; n.s.) or Treatment x Day interaction ($F(1,23) = 0.44$; n.s.). Thus, exposure to cues/context increased locomotion in the cocaine-expected condition compared to the saline-expected condition, but in contrast to USV results, there was no detectable enhancement after weekend cocaine/cue deprivation (see Fig. 11).

Extinction Sessions

50-kHz USVs

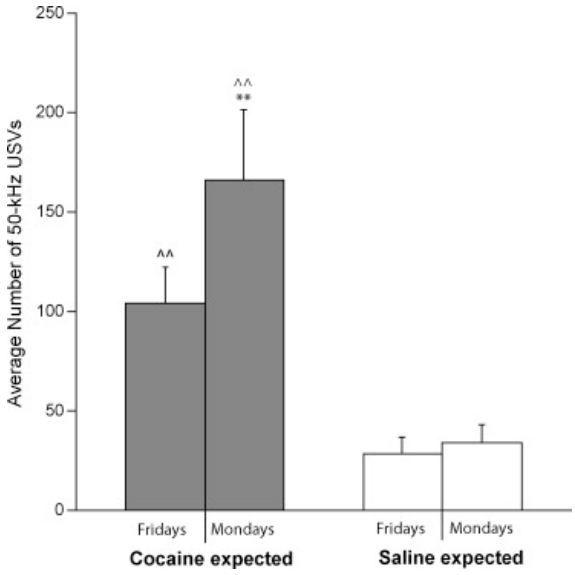
Two-way repeated measures ANOVA showed significant effects of Treatment ($F(1,21) = 8.36$; $p = 0.009$) and Day ($F(1,21) = 8.828$; $p = 0.007$), but no significant Treatment x Day interaction effects ($F(1,21) = 2.82$; n.s.). Extinction training significantly reduced cue-elicited USVs, but the level of calling remained higher in the cocaine-expected condition compared to the saline-expected condition. In addition, the level of calling in the cocaine-expected condition was significantly higher on Mondays compared to Fridays (see Fig. 12).

Locomotor Activity

Two-way repeated measures ANOVA showed significant Day ($F(1,21) = 5.519$; $p = 0.034$), but not Treatment ($F(1,21) = 1.32$; n.s.) or Treatment x Day interaction effects ($F(1,21) = 0.127$; n.s.). As shown in Fig. 13, cocaineand saline groups showed

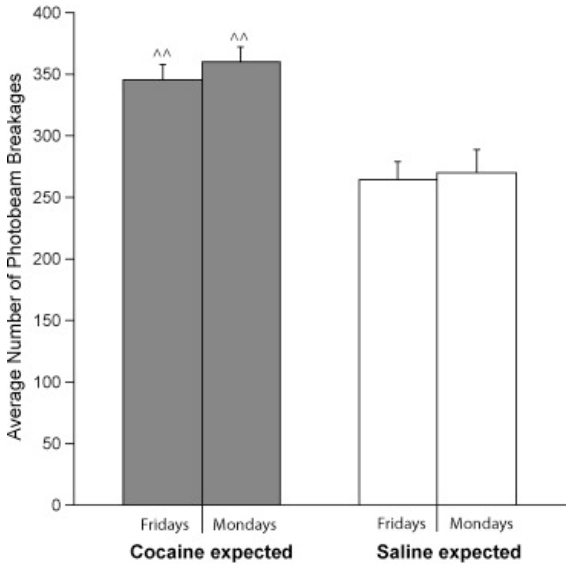
comparable levels of locomotion during extinction training and the effects of weekend deprivation were minor at best.

Figure 10: Cocaine and Cue Abstinence: 50-kHz USVs (Conditioning)



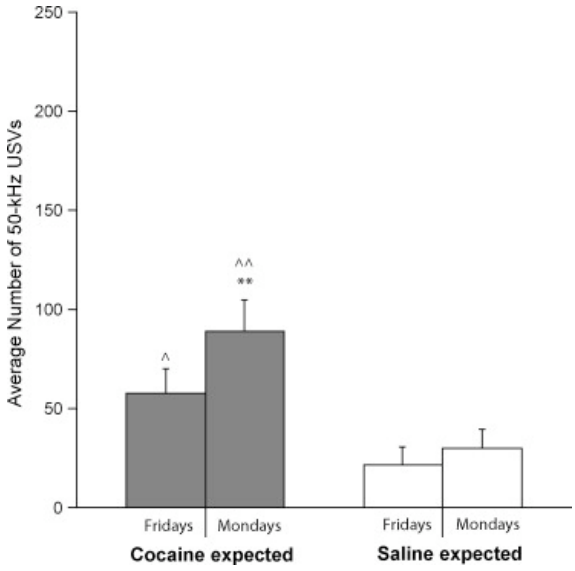
Expectation of forthcoming cocaine exposure, compared with saline expectation, was associated with a marked increase in USVs. This effect was significantly enhanced on Mondays (compared to Fridays) following a weekend of abstinence in the home cage. Thus, keeping rats away from cues and context for 2 days was sufficient to boost the level of calling once they returned to the conditioning chambers. **Significantly greater number of USVs on Mondays compared to Fridays; $p < 0.01$; paired samples t-test; ^^ Significantly greater number of USVs compared to matched saline day at $p < 0.01$; independent samples t-test.

Figure 11: Cocaine and Cue Abstinence: Locomotor Activity (Conditioning)



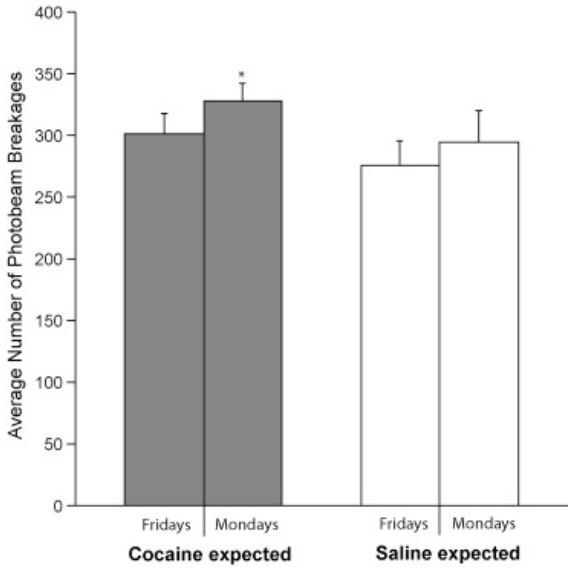
Cocaine-experienced animals showed significantly higher locomotor activity than the saline group on both Fridays and Mondays pre-drug intervals. ^^Significant difference compared to matched day saline values at $p > 0.01$; independent samples t-test. However, locomotor activity in the cocaine-experienced rats did not significantly differ as a result of 2 days of cocaine and cue abstinence.

Figure 12: Cocaine and Cue Abstinence: 50-kHz USVs (Extinction)



Extinction training reduced the number of cocaine-anticipatory USVs, but there were significantly more USVs on both Fridays and Mondays compared to the saline rats. ^,^^ Significant difference compared to matched day saline values at $p < 0.01$ and 0.05, respectively; independent samples t-test. In addition, enhancement of calling on Mondays relative to Fridays following weekend home cage confinement was still significant for the cocaine-experienced animals. **Significant difference at $p < 0.01$; paired samples t-test.

Figure 13: Cocaine and Cue Abstinence: Locomotor Activity (Extinction)



During extinction training, overall activity between the cocaine and saline groups was not significantly different, but the cocaine-expected condition had slightly enhanced locomotor activity following 2 days of cue and drug abstinence. *Significant difference at $p < 0.05$; paired samples t-test.

DISCUSSION

50-kHz USVs and locomotor activity were increased in anticipation of cocaine exposure and decreased in anticipation of extinction sessions. However, a brief period of home cage confinement (over weekends) during the cocaine conditioning phase substantially enhanced the number of cue-induced USVs (i.e., more calls on Mondays than on Fridays), but not locomotor activity. Thus, under these conditions, anticipatory vocalization counts were a more sensitive measure of the cocaine deprivation effect than were locomotor activity scores. Even during extinction training it was apparent that a 2-day confinement to the home cage potentiated the number of USVs when the animals were placed into the previously drug-paired operant chamber.

A close examination of the data revealed that the potentiation of USVs was significant even on the first Monday following a 2-day absence from the drug-paired environment and did not escalate with each successive abstinence interval. These findings indicate that learning through repeated abstinence experience was not required for the observed USV effect and further suggest that cocaine abstinence may lead directly to enhanced incentive salience of drug context. These data are in line with previous work reporting that alcohol- and nicotine-associated environments provoked relapse behaviors (Koob 2000; Lopez-Moreno et al. 2004; Mucha et al. 1999). However, our findings with cocaine conditioning are not fully consistent with conditioned heroin effects, as contextual cues associated with heroin withdrawal did not motivate drug seeking unless heroin reinforcement had been made available in the withdrawal state. Under these circumstances, learned associations between rescue from withdrawal symptoms and/or relief from conditioned withdrawal effects and a heroin-associated context appeared to

enhance relapse behaviors with successive returns to the heroin-associated environment (Hellemans et al. 2006).

Rats given free-access to alcohol followed by a period of imposed abstinence show a transient but pronounced increase in alcohol intake upon renewed alcohol availability, commonly termed the "alcohol deprivation effect" (Sinclair and Senter 1968). A similar effect may have been found with nicotine reinforcement (O'Dell and Koob 2007). Although no anticipatory behaviors were assessed in these studies, repeated cycles of deprivation and access to drugs increased the magnitude and duration of reinstated consumption (Bell et al. 2006; Rodd et al. 2004). These data have led to the paradoxical suggestion that, in drug-dependent individuals, avoiding drug cues or context when trying to quit may actually promote craving or obsession and perhaps impede continued drug abstinence. The expression of the alcohol deprivation effect is proposed to be an animal model of alcohol craving (Heyser et al. 1997) and has previously been used to investigate pharmacological treatments for preventing relapse (Heyser et al. 2003). Therefore, the measurement of USVs elicited during cocaine or other drug cue presentation may be useful for testing craving in animal studies and for exploring potential neural mechanisms of plasticity underlying learning and memory of hedonic events.

50-kHz USVs are also emitted in response to cues that have come to predict the opportunity to mate or self-stimulate the medial forebrain bundle (Bialy et al. 2000; Burgdorf et al. 2000; Ciucci et al. 2009; Ciucci et al. 2007), as well as during exposure to the side of a chamber in which morphine or amphetamine was delivered (conditioned place preference) (Knutson et al. 1999). This class of USVs also increases during drug exposure and remains high persistently with repeated experience, perhaps reflecting sensitization and an enhancement of the reinforcing properties of the drug and/or its

context (Ahrens et al. 2009; Mu et al. 2009). It is important to note that long 22-kHz calls, which have been linked to aversive events (Knutson et al. 2002), were not detected in any group of rats, not even during extinction sessions.

Drug obsession is an important characteristic of drug dependence (Vanderschuren and Everitt 2004). To the extent that the number of 50-kHz USVs in rats parallels drug cue salience and the potency of drug cues and context to engage drug expectation and incentive motivation, inclusion of this measure in pre-clinical studies may help to improve their relevance in translational drug dependence research.

Chapter 5: The Missing Variable: Ultrasonic Vocalizations Reveal Hidden Sensitization and Tolerance-Like Effects During Long-Term Cocaine Administration

ABSTRACT

Subtypes of 50-kHz ultrasonic vocalizations (USVs) in rats are thought to reflect positive affect and occur with cocaine or amphetamine delivery. In contexts predicting forthcoming cocaine, pre-drug anticipatory USVs are initially minimal during daily sessions but gradually escalate over several weeks, presumably as the animal learns to expect and look forward to impending drug access. To gain more insight into motivational aspects of cocaine intake in animal models of drug dependence studies, it is important to compare experience-dependent changes in lever response rate, USVs and locomotion during cocaine conditioning and extinction trials. To address whether cocaine-induced increases in lever responding and locomotor activity correspond with USV production. The study also determined whether short-term cocaine and context deprivation effects could be detected during conditioning or extinction. Rats underwent 20 days of 60-min sessions of self- or yoked administration of cocaine (0.75 mg/kg/infusion, i.v.), followed by 19 days of extinction training (8 weeks total, weekends off). Lever responding for cocaine and cocaine-induced locomotor activity increased across conditioning sessions. In contrast, the number of frequency modulated (FM) 50-kHz USVs evoked in response to cocaine infusion decreased with cocaine experience, suggesting perhaps tolerance to the rewarding properties of the drug. In addition, USVs but not lever pressing or locomotion are affected after brief periods of drug and/or drug context abstinence. Except for initial drug exposure, increased cocaine seeking during

cocaine delivery could reflect either enhanced drug motivation or the development of drug tolerance, but not enhanced positive affect.

INTRODUCTION

Cocaine is a drug with psychoactive and stimulating properties. The psychological effects of low doses of cocaine are described as enhanced euphoria, sense of well-being and self-esteem, accompanied by stimulating effects such as increased energy and mental alertness (Spotts and Shontz 1984). However, chronic cocaine use in humans has been reported to lead to a decrease of its pleasurable euphoric effects, accompanied by an increase in frequency of cocaine use and the administration of escalating cocaine doses (i.e., development of apparent drug tolerance) (Small et al. 2009). Increased doses eventually lead to the desired "high", but are usually accompanied by feelings of irritability, anxiety and paranoia (Breiter et al. 1997; Resnick et al. 1977; Spotts and Shontz 1984; Trinkoff et al. 1990; Trinkoff et al. 1989).

In animal models of drug dependence, the rewarding properties of cocaine have been inferred from observable animal behavior such as increased effort to obtain self-administered cocaine infusions and preference for cocaine-paired environments (Caine et al. 1995; Campbell et al. 2000; Hooks et al. 1994; Kalivas et al. 1988; McBride et al. 1999; Roberts et al. 1989; Spyraki et al. 1987; Zakharova et al. 2009). In addition, negative effects of cocaine have been observed in several animal studies, in which cocaine induces or enhances defensive behaviors, such as freezing, crouching and flight (Blanchard and Blanchard 1999; Blanchard et al. 2000; Blanchard et al. 1998), and cocaine delivery in a runway setup has been shown to trigger conflict behavior (e.g., approach/avoidance) in rats (Ettenberg and Geist 1993).

During episodes of major significance humans and animals have emotional reactions that involve responses such as physiological activation, motivational, perceptual, evaluative and learning processes (Peper 2006). Ultrasonic vocalizations

(USVs) emitted by rats in response to significant events in their environment can be used as real-time reflection of emotional status. For instance, food presentation and social encounters have been shown to cause an increase in the emission of high frequency ("50-kHz") calls (Burgdorf et al. 2000; Knutson et al. 1998), whereas the presence of a predator, aversive footshock or the touch by an unfamiliar human has been reported to elicit low frequency ("22-kHz") calls (Blanchard et al. 1991; Brudzynski and Ociepa 1992).

USVs have lately received increased attention in drug dependence studies because short-term administration of amphetamine or cocaine has been shown to evoke the emission of positive, in particular frequency modulated (FM), 50-kHz USVs (Ahrens et al. 2009; Barker et al. 2010; Browning et al. 2011; Mu et al. 2009; Simola et al. 2009; Williams and Undieh 2010; Wintink and Brudzynski 2001). Activation of dopaminergic (DA) transmission in the mesolimbic DA system by central amphetamine and glutamate administration as well as by electrical stimulation of the brain (e.g., nucleus accumbens, ventral tegmental area) increases the emission of 50-kHz calls (Burgdorf et al. 2001; Burgdorf et al. 2007; Fu and Brudzynski 1994). Furthermore, increased emissions of 50-kHz USVs have been reported in environments associated with cocaine, morphine and amphetamine administration and in response to cues that indicate impending drug availability (Knutson et al. 1999; Ma et al. 2010; Maier et al. 2010). These effects are of particular relevance since cocaine-induced USV data of the same animals emitting cocaine-anticipatory USVs (Ma et al. 2010; Maier et al. 2010) are shown here.

The two main goals of this study were to examine 1) hypothesized changes in cocaine-induced USVs during relatively long-term administration and extinction training sessions, and 2) how they correlate with common measures, such as locomotor activity and lever responding for intravenous self-administration of the drug. Our results may

help to further understand the development of cocaine dependence and to lead to improvements in modeling drug dependence.

MATERIALS AND METHODS

Animals

Five-week old male Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were obtained for this study. During handling and lever response training (four weeks total) the rats were group-housed in polypropylene cages. After surgery, the animals were single-housed. All animals were kept under a reversed 12:12 light/dark cycle (lights on at 8 p.m.). Only during daily handling, after lever response acquisition and recovery from surgery, was food provided *ad libitum*. At all other times, the animals were food restricted to maintain body weight. Animals were tested daily between 9 a.m. – 12 p.m., with weekends off.

Apparatus

Lever response training, conditioning and extinction sessions were conducted in single-lever operant chambers (28 cm x 22 cm x 21 cm). The operant chambers were located within sound-attenuating boxes (Med-Associates, St. Albans, VT) to minimize external noise and light. During conditioning, catheterized rats were intravenously connected via tubing to a sterile cocaine (0.75 mg/kg/injection) or saline syringe (0.1ml/injection) mounted on a motorized pump (Razel Scientific Instruments, Model A, St. Albans, VT). Each lever response during conditioning and extinction sessions resulted in a 6-second infusion of either cocaine or saline solution, accompanied by the blinking of a stimulus light above the lever (i.e.,15 times during the 6-sec infusion). Three sets of

photobeams, two positioned 5 cm from each sidewall and one in the center, were used to assess locomotor activity. Ultrasonic microphones (PCB Piezotronics, Buffalo, NY; working frequency range: 5 - 126,000 Hz) were positioned in the center of the operant chamber with a maximal distance of 22 cm to ensure digital recording of USVs. Photobeam interruptions and USVs were assessed for the entire session in 10-minute intervals.

Self-Administration and Yoked Groups

The rats were randomly assigned to one of the following four groups: cocaine self-administration (SA), cocaine yoked (Y), saline SA and saline Y. Only SA groups had access to the lever during operant training and experimental sessions. Yoked animals did not have access to the lever at any time during the experiment. Each SA rat was paired with a Y rat that passively received identical amounts of food pellets and cocaine or saline infusions.

Lever Response Training

SA rats underwent lever response training for sucrose pellets (45 mg, P.J. Noyes, Lancester, NH) on a fixed ratio-1 (FR-1) reinforcement schedule. Animals were trained for lever response for 12 days in 10-minute sessions. Lever presses resulted in the delivery of a sucrose pellet cued by a short illumination of a stimulus light above the lever. Each Y animal received the identical amount of sucrose pellets as its counterpart, with no lever being available.

Jugular Catheterization Surgery

The catheterization surgical procedure was performed as previously described (Depoortere et al. 1993). Briefly, all animals were surgically implanted with a catheter made out of Silastic tubing (8.5 cm, 0.64 mm o.d.), of which one end was connected to a cannula endpiece (Plastics One, Roanoke, VA). Anesthesia was maintained and delivered through a gas delivery system (VetEquip, Inc, Pleasanton, CA) and consisted of an isoflurane (2.5 - 4 %; AErrane, Baxter Healthcare, Deerfield, IL) oxygen (0.8 l/min; Airgas Southwest, Corpus Christi, TX) mixture. After the catheter was inserted into the right jugular vein and secured with surgical suture, its connected end-piece was subcutaneously guided towards an incision on top of the head. Before closing the jugular incision, three drops of the antibiotic gentamicin sulfate (50 mg/ml) were applied to inhibit infection. The catheter was fixed on the skull with four stainless steel anchor screws (Plastics One, Roanoke, VA) embedded in acrylic cement. The anti-inflammatory and analgesic drug Carprofen (5 mg/kg) was also administered subcutaneously. 0.1 ml of the antibiotic Timentin (100 mg/1.5 ml), diluted in heparinized saline (1 U/ml heparin), was intravenously administered to the animals during the recovery period. Conditioning sessions commenced after a one-week recovery from the surgery. Heparinized saline (0.1 ml, i.v.) was daily administered to the animals to maintain catheter patency and to test catheter function.

Conditioning and Extinction Sessions

Conditioning and extinction sessions were conducted over 4 weeks each (i.e., 8 weeks total), 5 days per week, resulting in 20 days of conditioning and 19 days of extinction sessions. Stimulus conditions were the same between conditioning and extinction sessions except that all animals received non-reinforcing saline infusions

during extinction training. Sessions commenced as follows: Animals were placed into dark operant chambers for 10 minutes prior to drug availability for assessment of drug/saline anticipatory USVs as previously reported (Ma et al. 2010; Maier et al. 2010). (Please note that more animals were added to the experiment after the two previously published studies were submitted for publication.) After the pre-drug interval, the house light was illuminated and olfactory as well as visual cues (rose or cinnamon scents and black or white wall covering) were introduced to the animals. For the next 60 minutes, the lever was available for fourteen 30-second intervals only for the SA groups. During those intervals (at time points 610s, 700s, 1060s, 1310s, 1760s, 2070s, 2160s, 2520s, 2770s, 3220s, 3530s, 3710, 3900s, 4060s), the stimulus light located above the lever slot was illuminated for SA and Y animals. Lever response (FR-1) of the SA animals resulted in the administration of either sterile cocaine or saline solution to themselves and their yoked counterparts (see more details under 'Apparatus'). Animals were kept in their home cages over the 2-day weekend to determine whether drug and/or cue/ test context deprivation behavioral effects (enhanced lever responding, USVs and/or locomotion) might be observed upon relief from a brief abstinence.

Data Collection

Vocalization (USVs), locomotor and lever response behaviors during the 60-minute period of cocaine or saline administration of each experimental session were recorded and analyzed. MED-PC software (Med Associates, St. Albans, VT) was used to detect locomotion and lever responses, and ultrasonic microphones to detect rat USVs. USVs were recorded and assessed with RECORDER multi-channel and SASLab Pro software (Avisoft Bioacoustics, Berlin, Germany). Recording settings were as follows:

Sampling rate: 22050 Hz; Format: 16 bit; Buffer: 0.2 s; Range: 40 % 250 kHz; FFT size: 256; Resolution: 86 Hz.

Assessment of Rat USVs

Detected USVs were assessed using SASLab Pro software as follows: Each recorded 10-minute interval was converted to a spectrogram and imported to a data file. Trained data analyzers used visual and auditory confirmation to assess the number of verified USVs. Experimenters performing USV differentiation were blind to the experimental groups and were individually trained by Esther Maier to assure consistent analyses. The proportion of flat/FM 50-kHz USVs were determined from a data subset consisting of one session per week (8 total) in 3 animals per group. Flat calls were defined as those varying < 5 kHz (as displayed on software-generated spectrogram) and lacking detectable auditory frequency variation in playbacks set at 11.025 kHz. Flat calls were determined to equal less than 1% of total calls across all reviewed sessions, therefore total USVs reported here include both flat and FM USVs. Additional assessment methods can also be observed in video format (Maier et al. 2010). It is important to mention that the co-occurrence of USVs and light cue could not be determined due to technical limitations.

Drug

Cocaine HCl (0.75 mg/kg/injection; RTI International, Triangle Park, NC) was dissolved in sterile 0.9 % sodium chloride. Animals were weighed daily to assure a cocaine dose of 0.75 mg/kg body weight per infusion delivered immediately after each lever response.

Data Analyses

USVs and locomotor activity recorded during the 60-minute conditioning and extinction sessions were analyzed separately using three-way repeated ANOVAs [Drug Condition (cocaine, saline) x Mode (self-administered vs. yoked) x Session Days (1-20 or 21-39)] (data for one SA cocaine and one Y cocaine animal were assessed during conditioning but not during extinction). Two-way ANOVAs were conducted on lever response data, and one-way ANOVAs were performed to examine within-group changes associated with cocaine and saline experience and to monitor weekly changes in USV-eliciting effects of cocaine injections (USVs/lever responses). To evaluate the effects of cocaine and cue deprivation during conditioning and extinction sessions, paired samples t-tests were used to compare USVs, lever responses and locomotor activity between Fridays (i.e., days 5, 10, 15 and 25, 30, 35) and the following Mondays (i.e., days 6, 11, 16 and 26, 31, 36) within cocaine and control groups. Independent samples t-tests were used to compare these values between the cocaine and control conditions. Posthoc analyses (Least Significant Difference tests) to determine precise between- and within-group differences were performed in the event of significant interaction effects. Levene's Test for Equality of Variances was performed for all data sets. When equality of variance was violated, log transformations were applied and analysis of variance was conducted on the transformed data (i.e., for '50-kHz USVs during Conditioning', 'Locomotor activity during Conditioning', 'USV/Lever Response Ratio during Conditioning' and '50-kHz USVs during Extinction'). Pearson Correlation analyses were conducted to determine various relationships between food-conditioned USVs (i.e., food anticipatory USVs on Day 1 of cocaine conditioning) and weekly USV/Lever Response Ratios. Data

lost due to equipment recording errors (i.e., 1.5 % of total USVs and less than 1 % of locomotor activity and lever response measures) were replaced as mean group values.

RESULTS

Conditioning Sessions

Lever Responses during Conditioning

Two-way repeated measures ANOVA between SA cocaine and SA saline groups showed significant Drug ($F(1,12) = 5.415$; $p = 0.038$), Day ($F(19,228) = 4.703$; $p < 0.001$) and Drug x Day interaction effects ($F(19,228) = 4.326$; $p < 0.001$). One-way ANOVAs revealed significant within-group Day effects in SA cocaine ($F(19,152) = 11.71$; $p < 0.001$), but not the SA saline group ($F(19,95) < 1.0$; n.s.). Posthoc tests showed that saline control animals performed significantly more lever responses than cocaine rats during the first 2 sessions. However, significantly higher lever responses for the cocaine animals were observed during the last two weeks of sessions (see Fig. 14a).

50-kHz USVs during Conditioning

Three-way repeated measures ANOVA showed significant Drug ($F(1,23) = 142.22$ $p < 0.001$) and Day ($F(19,437) = 2.73$; $p = 0.001$), but no significant Mode or Drug x Day interaction effects. One-way ANOVAs performed on data from combined cocaine and control groups (e.g., SA and Y groups for each drug condition) revealed significant within-group Day effects for the cocaine ($F(19,285) = 3.57$; $p < 0.001$), but not the saline control groups ($F(19,190) < 1.0$; n.s.) (see Fig. 15a).

Locomotor Activity during Conditioning

Three-way repeated measures ANOVA showed significant effects of Drug ($F(1,23) = 201.7$; $p < 0.001$), Day ($F(19,437) = 5.36$; $p < 0.001$) and Drug x Day interaction ($F(19,437) = 4.73$; $p < 0.001$) effects, but not Mode or any other interaction effects. One-way ANOVAs revealed significant Day effects for the cocaine-receiving groups ($F(19,285) = 11.55$; $p < 0.001$), but not the saline control conditions ($F(19,190) < 1.0$; n.s.). Significantly greater levels of locomotor activity in both cocaine groups compared to the saline control groups at every tested session were shown. (see Fig. 16a).

USV/Lever Response Ratio

To compare weekly changes in the number of USVs elicited per cocaine or saline injection, one-way repeated measures ANOVA were performed on USVs/lever response ratio data (number of USVs per 0.75 mg/kg cocaine or 0.1 ml saline in SA cocaine and saline groups) from rats with at least 3 lever responses/week. Significant week effects ($F(3,18) = 11.038$; $p < 0.001$) were revealed in SA cocaine ($n = 7$) rats but not SA saline ($n = 6$) animals ($F(3,15) < 1.0$; n.s.), reflecting the significant decrease in USVs/lever response ratio from the first two weeks compared to the last two weeks in the SA cocaine, but not SA saline groups. (see Fig. 17a).

USVs, Lever Responses and Locomotor Activity before and after 2-Day Cocaine Abstinence Intervals during Conditioning

T-tests of USV, locomotor activity and lever response data between Fridays and the following Mondays were performed to determine the effects of 2-day cocaine and cue abstinence. Since no significant effects of Mode on USVs and locomotor activity were

detected during conditioning sessions, SA and Y groups were combined for these comparisons. USVs for cocaine, but not saline groups, were significantly higher on Mondays compared to Fridays ($t(47) = 4.8$, $p < 0.001$; Paired samples t-tests) and significantly greater than USVs in the saline groups (Monday: $t(79) = 5.23$; Friday: $t(79) = 4.40$; $p < 0.001$ for both; Independent samples t-test) (see Fig. 5a). Lever responses in cocaine-reinforced animals (i.e., SA animals) were significantly greater than SA saline controls on Fridays ($t(42) = 2.941$; $p = 0.005$) and Mondays ($t(43) = 2.783$; $p = 0.008$), but neither group showed significant 2-day abstinence effects ($t(26) = 1.75$ and $t(16) = 0.534$ for cocaine and saline, respectively; n.s. for both) (see Fig. 5b). Locomotor activity was significantly higher in the cocaine compared to saline groups on Friday ($t(78) = 8.955$; $p < 0.001$) and Monday ($t(79) = 9.364$; $p < 0.001$). However, comparisons between Fridays and Mondays in the cocaine ($t(46) = 1.42$; n.s.) and saline ($t(32) = 0.429$; n.s.) groups showed no significant abstinence effects on locomotion (see Fig. 19c).

Correlational Analyses: Food-conditioned USVs and USV/Lever Response Ratio

For the SA cocaine group, food-conditioned USVs prior to the first cocaine injection on Day 1 (10 min) were significantly correlated with USV/lever response ratios for Weeks 1, 2 and 4 ($R = 0.711$, 0.795 and 0.829, respectively; $p < 0.05$ and 0.01, Pearson Correlation), but there were no significant correlation in all the other weeks (see Fig. 20). USVs in the SA Saline group were not significantly correlated with lever responses during self-administration sessions

Extinction Sessions

Lever Responses during Extinction

Two-way repeated measures ANOVA showed significant Drug ($F(1,12) = 75.231$; $p < 0.001$), Day ($F(18,216) = 12.326$; $p < 0.001$) and Drug x Day interaction effects ($F(18,216) = 6.222$; $p < 0.001$). One-way ANOVAs revealed significant within-group Day effects in SA cocaine ($F(18,126) = 15.61$; $p < 0.001$), but not the SA saline group ($F(18,90) < 1.0$; n.s.). Animals with previous cocaine self-administration experience maintained significantly greater lever response rates compared to controls across virtually all extinction sessions (i.e., 18/19 sessions) (see Fig. 14b).

50-kHz USVs during Extinction

Three-way repeated measures ANOVA showed significant Drug ($F(1,21) = 25.04$; $p < 0.001$) and Day ($F(18,378) = 2.76$; $p < 0.001$), but no other significant interactions. One-way ANOVAs revealed significant Day effects in rats that had previously received cocaine during conditioning (e.g., SA and Y cocaine groups; $F(18,234) = 2.95$; $p < 0.001$), but not in animals with no previous cocaine experience (e.g., saline control conditions; $F(18,180) = 1.31$; n.s.) (see Fig. 15b).

Locomotor Activity during Exinction

Three-way repeated measures ANOVA showed significant Day ($F(18,378) = 3.027$; $p < 0.001$) and Drug x Day interaction effects ($F(18,378) = 1.797$; $p = 0.024$), but no Drug, Mode ($F(1,21) = 3.522$ and 2.066, respectively, both n.s.) or other interaction effects. One-way ANOVAs revealed significant Day effects for animals with previous cocaine experience ($F(18,234) = 4.15$; $p < 0.001$), but not saline control conditions

($F(18,180) < 1.0$; n.s.). Locomotor activity significantly decreased in cocaine, but not saline groups, from the first to last extinction session (see Fig. 16b).

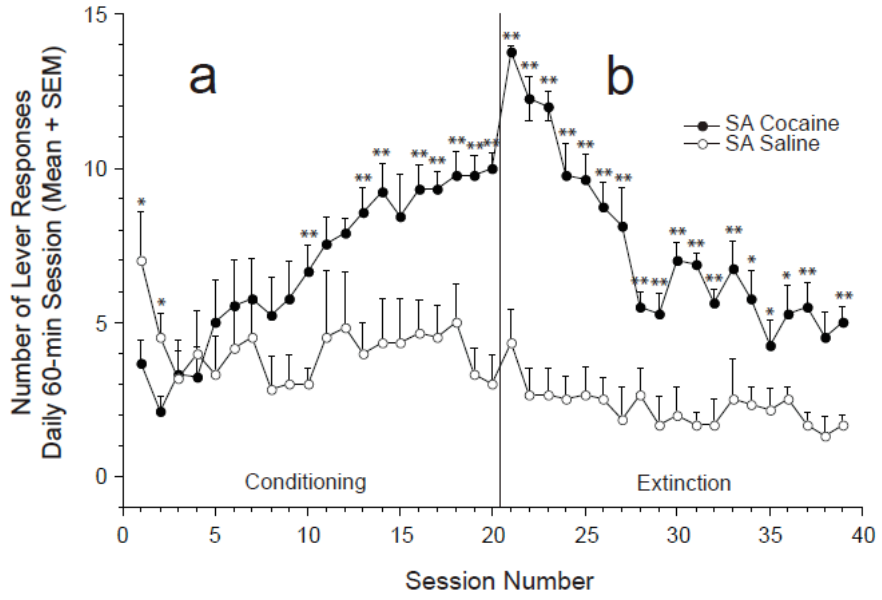
USV/Lever Response Ratio during Extinction

One-way ANOVAs performed on USV/Lever responses (number of USVs per 0.1 ml saline in SA cocaine and saline groups) from rats with at least 3 lever responses/week showed no significant differences across Weeks for SA cocaine (n = 7) or SA saline (n = 6) groups ($F(3,18) = 2.05$ and $F(3,15) < 1.0$, respectively; both n.s.) (see Fig. 17b).

USVs, Lever Responses and Locomotor Activity before and after 2-Day Cue Abstinence Intervals during Extinction

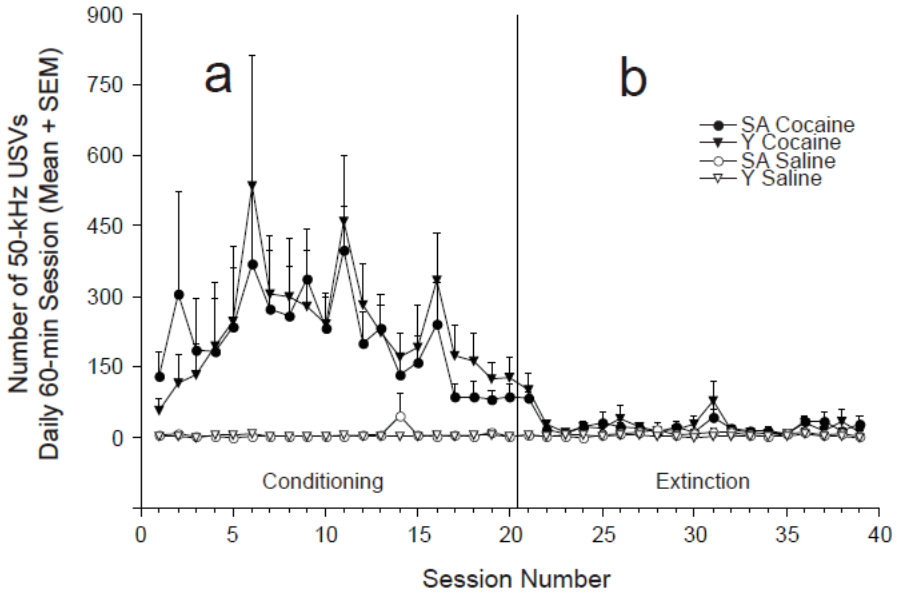
T-tests were performed on USVs, lever responses and locomotor activity occurring on Fridays and the following Mondays to determine effects of 2-day cue abstinence in cocaine-experienced and cocaine-naïve groups. Cocaine-experienced rats showed significantly greater USVs ($t(41) = 2.83$; $p = 0.007$) and locomotor activity ($t(41) = 2.96$; $p = 0.005$) when placed back into the operant chamber after 2 days of home cage only. USVs were significantly greater in cocaine-experienced versus cocaine-naïve rats on Fridays ($t(73) = 2.31$; $p < 0.001$) and both USVs and locomotor activity were significantly greater in cocaine rats on Mondays (USVs: $t(73) = 3.31$; $p < 0.001$; locomotor: $t(73) = 2.032$; $p = 0.046$) (see Fig. 6a and c). Lever responses by both cocaine-experienced ($t(23) = 0.00$; n.s.) and cocaine-naïve ($t(17) = 0.156$; n.s.) rats did not differ on these days (see Fig. 19b).

Figure 14: Lever Responses



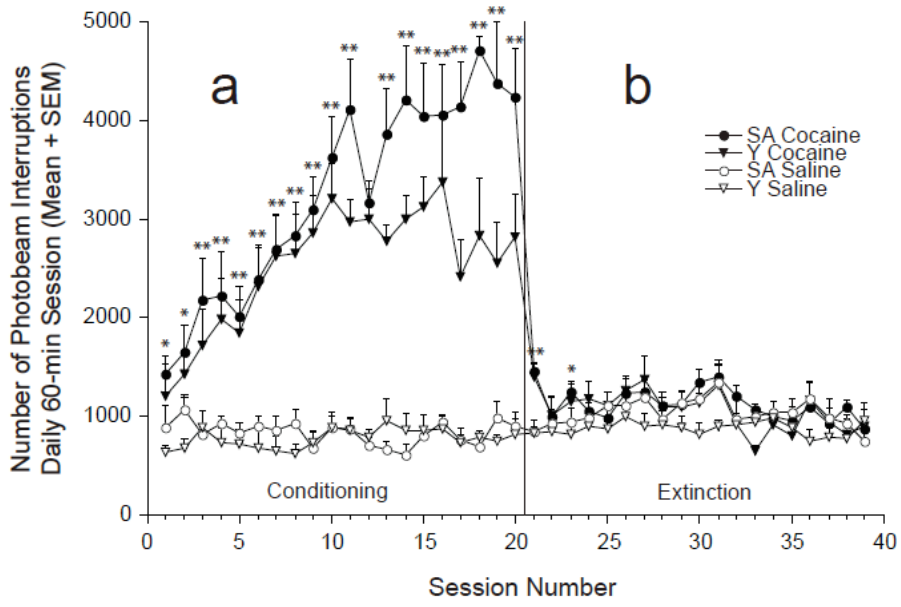
Lever responses (mean + SEM) during conditioning and extinction sessions: Self-administration (SA) groups. Response rates of the SA cocaine group across conditioning and extinction sessions were significantly greater than the SA saline (control) group. a. During the first 2 conditioning sessions (sessions 1-20), control animals (n = 5) showed significantly greater lever responses than SA cocaine rats (n = 9), but cocaine-reinforced responding gradually increased to significantly greater levels by the 10th session. b. Across all extinction sessions (sessions 21-39), cocaine-experienced animals (n = 8) maintained significantly greater lever response rates compared to saline controls (n = 5). *, ** = significantly greater compared to matched session between groups; $p < 0.05$ or $p < 0.01$, respectively.

Figure 15: 50-kHz USVs



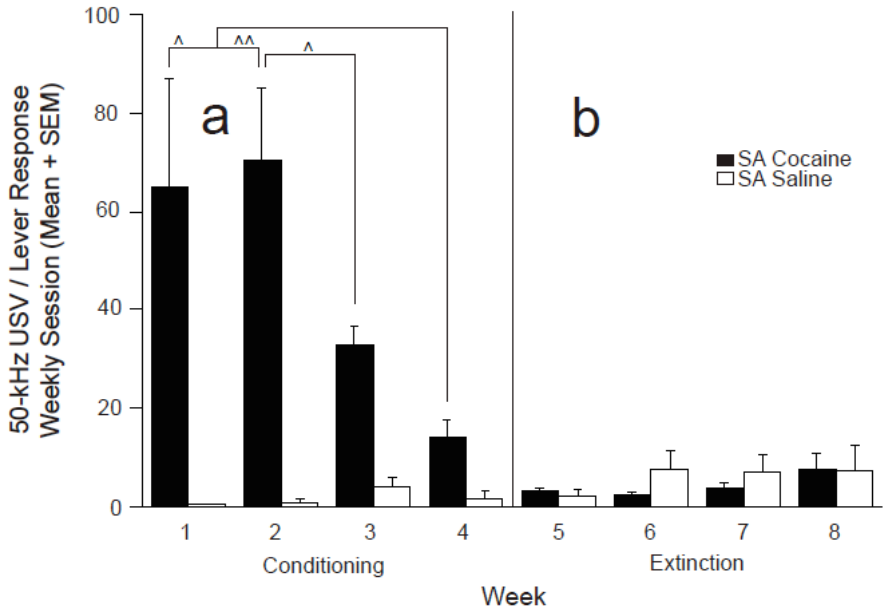
50-kHz USVs (mean + SEM) during conditioning and extinction sessions: Self-administering (SA) and Yoked (Y) groups. a. Daily 50-kHz USVs were significantly greater in the cocaine groups (SA cocaine, n = 9; Y cocaine, n = 7) compared to saline groups (SA saline, n = 6; Y saline, n = 5). No significant differences were detected between SA and Y groups. b. Cocaine-experienced animals (SA cocaine, n = 8 and Y cocaine, n = 6) emitted significantly more 50-kHz USVs than saline controls (SA saline, n = 6 and Y saline, n = 5) over extinction training, but remained at control levels for the majority of the sessions.

Figure 16: Locomotor Activity



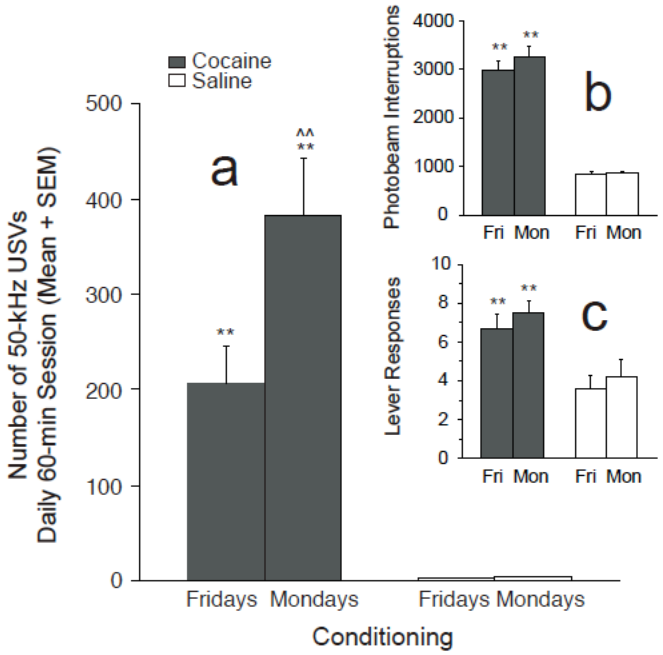
Locomotor activity (mean + SEM) during conditioning and extinction sessions: SA and Y groups. a. Across all conditioning sessions, mean activity scores were significantly greater in cocaine groups (SA cocaine, n = 9; Y cocaine, n = 7) compared to controls (SA saline, n = 6; Y saline, n = 5), with no significant differences within SA and Y subgroups of each condition. b. During extinction training, a significant decrease from the first to last session was detected in the cocaine, but not saline groups. *, ** = significantly greater activity levels for both cocaine groups compared to both saline control conditions during the same session; $p < 0.05$ or $p < 0.01$, respectively.

Figure 17: USV/Lever Response Ratio



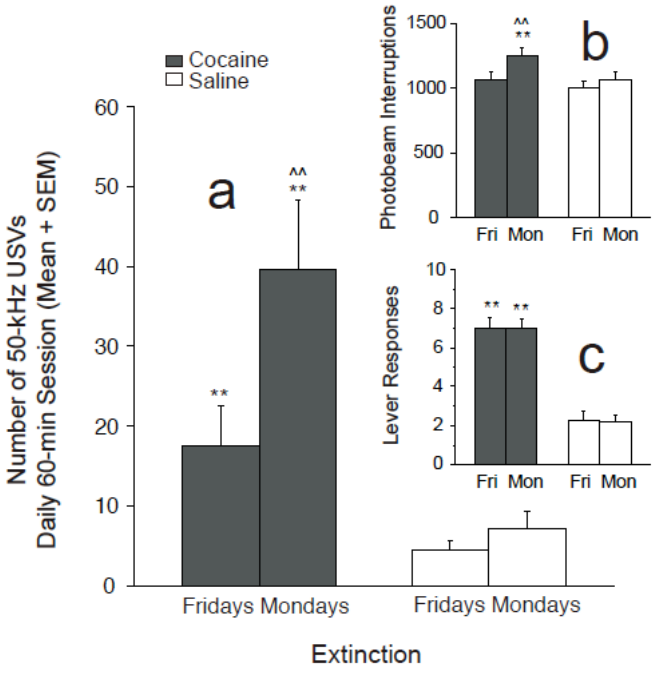
USV/Lever response ratio (Weekly means + SEM) during conditioning and extinction: SA groups. a. Including rats eliciting at least 3 lever responses/week, the number of 50-kHz USVs induced by each unit dose of cocaine (0.75 mg/kg; n = 7) decreased significantly during the last 2 weeks of conditioning compared to the first two weeks. The USV/lever response ratio across all four weeks of conditioning was comparable in control animals receiving the same volume of saline injections (e.g., 0.1 ml; n = 6) during each lever b. No significant differences in weekly USV/lever response ratios were detected between SA cocaine (n = 7) and SA saline (n = 6) groups during extinction sessions. ^, ^^ = significantly smaller within-treatment effect; $p < 0.05$ or $p < 0.01$, respectively.

Figure 18: Weekend-Effects during Conditioning



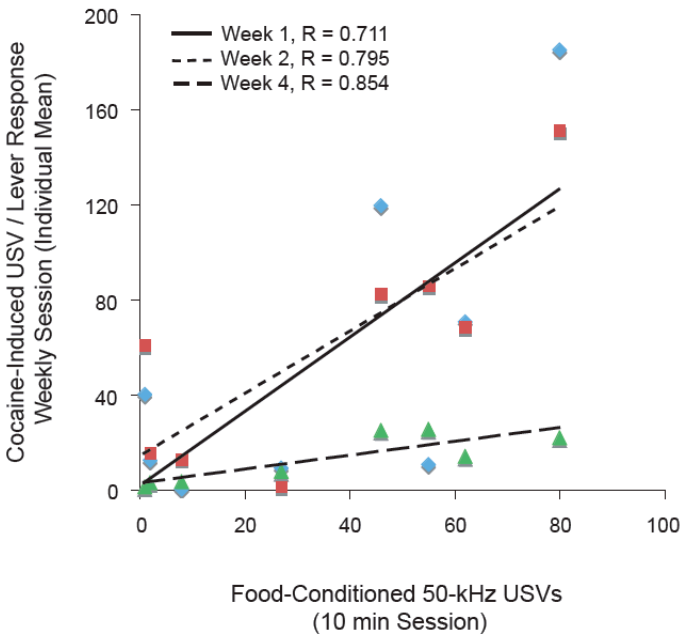
50-kHz USVs, locomotor activity and lever responses (means + SEM) before and after "weekend" cocaine and cue abstinence during conditioning sessions: SA and Y combined. a. Significantly greater cocaine-induced 50-kHz USVs (n = 16) were detected on the Mondays following 2-day cocaine and cue abstinence (e.g., remaining in home cage over weekend) compared to the 5th day of consecutive cocaine administration sessions (e.g., Fridays). No significant differences were detected in cocaine-naïve animals (n = 11) after identical home cage confinement. b and c. Locomotor activity and lever responses in the cocaine animals were significantly greater than saline groups on Friday and Monday sessions, but no group showed significant differences between sessions in these measures. ^^= significantly greater within-treatment effects on Mondays vs. Fridays; $p < 0.01$; ** = significantly greater than saline control condition on same day; $p < 0.01$.

Figure 19: Weekend-Effects during Extinction



50-kHz USVs, locomotor activity and lever responses (means + SEM) before and after "weekend" cue abstinence during extinction sessions: SA and Y combined. a. Significantly greater cocaine-induced 50-kHz USVs (n =14) were detected on the Mondays following 2 days of cue abstinence (e.g., weekend in home cage only) compared to the 5th day of consecutive extinction sessions (e.g., Fridays). No significant differences were detected in cocaine-naïve animals (n = 11) after identical home cage confinement. b. Cocaine-experienced rats showed significantly greater locomotor activity on Mondays compared to Fridays and compared to saline controls. c. Lever responses in the cocaine animals were significantly greater than saline groups on Friday and Monday sessions, but lever responses were comparable between sessions. ^^ = significantly greater within-treatment effects on Mondays vs. Fridays; $p < 0.01$; ** = significantly greater than saline control condition on same day; $p < 0.01$.

Figure 20: Relationship between and USV/Lever Response Ratio



SA Cocaine. A significant positive correlation was revealed between the number of 50-kHz USVs elicited during the 10-min anticipatory period on Day 1 (food-conditioned USVs) and USV/lever response ratio during Weeks 1, 2 and 4 (Pearson Correlation), but not during other weeks; $p < 0.05$ for Week 1 and 2, $p < 0.01$ for Week 4.

DISCUSSION

During the course of cocaine conditioning, both the number of lever responses and locomotor activity gradually escalated in the cocaine groups over several weeks, as expected. However, although cocaine-induced 50-kHz USVs also escalated during the first week of cocaine administration, it reached a plateau in the second week and gradually dropped off over the last two weeks despite the continuing escalation of lever responses for cocaine. Indeed, analyses of the number of USVs elicited per cocaine injection were significantly higher in the first two weeks, compared to the last two weeks during conditioning. Moreover, for the cocaine groups the number of 50-kHz USVs was significantly elevated on the session following 2-day (weekend) abstinence, but locomotor activity and lever response rates were not. Interestingly, the number of food-conditioned 50-kHz USVs elicited on the first day of and prior to cocaine exposure was significantly correlated with weekly cocaine-induced USV/lever response ratios during 3 out of 4 conditioning weeks. Thus, the emission of conditioned USV to food environments predicted the magnitude of the rewarding properties of cocaine.

Our study confirms previous findings that cocaine administration induces 50-kHz USVs in rats (Barker et al. 2010; Browning et al. 2011; Mu et al. 2009; Williams and Undieh 2010), as has been found with amphetamine (Ahrens et al. 2009; Barker et al. 2010; Burgdorf et al. 2001; Simola et al. 2009; Wright et al. 2010) but not caffeine (Simola et al. 2009). As previously reported (Maier et al. 2008), animals in the cocaine self-administration group progressively increased lever responding across conditioning sessions. These findings are in contrast to other limited-access studies (i.e., 1-hour sessions) that show little or no enhancement of drug intake behavior (Ahmed and Koob 1998; Ben-Shahar et al. 2004; Mantsch et al. 2004). Increased cocaine intake has mainly

been demonstrated in extended-access studies (i.e., > 6 hours) under FR conditions (Ahmed and Koob 1998; Ben-Shahar et al. 2004; Mantsch et al. 2004) as well as using progressive ratio procedures (Depoortere et al. 1993; Liu et al. 2005). In addition, an increase of cocaine-induced locomotor activity was evident throughout conditioning, which progressively increased with cocaine experience. While this finding is consistent with other reports of cocaine-induced behavioral sensitization after repeated administration of equal doses (Crombag et al. 2002; Hooks et al. 1994; Kalivas et al. 1988; Post and Rose 1976; Williams and Undieh 2010), the daily drug dose was not constant but rather regulated by the lever responses of the SA animals. Our study does not specifically address the development of sensitization effects, since animals increased cocaine intake as well as locomotor activity across sessions. A similar increase in cocaine-induced locomotor activity during self-administration was previously reported in our laboratory (Maier et al. 2008), as well as for cocaine-induced stereotyped activity (Lecca et al. 2007). Cocaine-induced stereotypy could also explain missing sensitization effects of cocaine, leaving stereotyped movements undetected (due to technical limitations in our experiment).

However, it is quite notable that over the 20 days (4 weeks, weekends off) of cocaine conditioning, the ratio of FM 50-kHz USVs per lever response (i.e., drug intake) was significantly lower during the last 2 weeks of conditioning, compared to the first 2 weeks. Since FM 50-kHz and not flat 50-kHz USVs are thought to be strongly associated with a reward state (Ahrens et al. 2009; Burgdorf et al. 2008; Burgdorf et al. 2007; Simola et al. 2009; Wohr et al. 2008; Wright et al. 2010), the observed decline in FM 50-kHz USVs, despite increased drug intake suggests a tolerance-like response developing to the rewarding properties of cocaine parallel to a sensitization to the reinforcing effects of the drug. However, since positive affect USVs can also be decreased by the presence of

aversive stimuli (Knutson et al. 1998), alternate explanations are also conceivable. For instance, it has been reported that cocaine in high doses can cause aversive states in human users (Spotts and Shontz 1984). As observed in the present study, there was a close correspondence between increasing levels of cocaine intake and the significant decrease in the number of 50-kHz USVs emitted per each cocaine injection. Although seemingly counter-intuitive (i.e., decrease in rewarding state despite higher drug intake), the higher cocaine intake seen in the last several cocaine sessions could have caused an increase in the non-rewarding effects of cocaine, resulting in fewer cocaine-induced USVs.

Nevertheless, with or without increased drug intake, a crucial factor likely contributing to our present findings concerns long-term effects of drug exposure. For example, even though previous work has reported that experimenter-delivered i.p. cocaine-, (Mu et al. 2009; Williams and Undieh 2010) or i.v. amphetamine-induced (Ahrens et al. 2009) USVs progressively increase with repeated administration of a constant drug dose, these studies were conducted over short intervals (5 days). However, Browning and colleagues recently published a long-term SA cocaine study that shows an increase of 50-kHz USVs during long-term cocaine self-administration (Browning et al. 2011). USVs were measured at four different time points during the experiment, in which the recordings were started immediately after the session commenced without a preceding anticipation period. This is of particular interest since recent publications from our lab (Ma et al. 2010; Maier et al. 2010) showed that during a drug-free 10-minute interval, the same rats used in the current study chronically emitted anticipatory 50-kHz USVs prior to cocaine self- or yoked administration that gradually and markedly increased over the entire 20 days of conditioning (Ma et al. 2010). Taken together, the eventual decrease in cocaine-induced USVs during cocaine availability may imply that a

gradual temporal shift of cocaine reward occurred, from the reward itself to the conditioned environment, as observed in people and non-human primates [see review, (Schultz 2010)].

Following four weeks of cocaine conditioning all rats underwent an additional four weeks of extinction training. A rapid decline in 50-kHz USVs occurred and the number of calls dropped down to control levels only two days into extinction. Note that even prior to the start of extinction training, the number of USVs elicited by each cocaine injection reduced markedly. Thus, the process that decreased the rewarding properties of cocaine already appeared to be underway. This process may have combined with extinction learning to inhibit reward and/or expectancy. Interestingly, a comparably rapid or extensive drop was not evident for lever response behavior. Throughout the entire extinction training, cocaine-experienced SA animals made substantially more non-reinforced lever responses than SA saline controls. These data suggest that lever responses in these animals were perhaps the result of an over-learned motor behavior rather than via motivational processes (Horvitz 2001). Alternatively, the high number of lever responses might reflect "checking"; a behavior that exists despite diminished expectations of changes in outcome (Djodari-Irani et al. 2011; Schwabe and Wolf 2011). In this case, the complete decline in USVs suggests that cocaine delivery was not anticipated though lever responding was persistent. Indeed, our SA saline rats showed a similar pattern during the first two days of conditioning; 50-kHz USVs at low levels during high rates of lever responding. Taken together, USVs may be a more sensitive measure of changes in reward conditions, and may prove to be a vital assessment tool in animal studies of cocaine dependence.

During cocaine conditioning, there was no effect of mode of drug administration (i.e., self- or experimenter administration) on the emission of FM 50-kHz USVs. Mode of

drug administration has been shown to elicit different cocaine effects, such as cocaine and cocaine-associated taste cue avoidance, release of glutamate in the ventral tegmental area and dopamine receptor levels (Stefanski et al. 2007; Twining et al. 2009; You et al. 2007). However, our current findings may imply that the mechanisms that trigger cocaine-induced FM 50-kHz USVs under the experimental conditions utilized here are independent of the motivational effects associated with cocaine delivery.

Note that in the present study, negative-affect 22-kHz calls were not observed in SA or Y animals during conditioning or extinction conditions. Others have reported emission of long flat 22-kHz calls during withdrawal from chronic cocaine exposure, but only when triggered by startle conditions (Mutschler and Miczek 1998; Mutschler and Miczek 1998). Long flat 22-kHz USVs are generally known to reflect a negative emotional state (Blanchard et al. 1991; Brudzynski and Ociepa 1992; Knutson et al. 2002). In a recent study utilizing 6-hr cocaine access sessions, short 22-kHz USVs were observed in rats self-administering two different cocaine doses (0.355 and 0.71 mg/kg/infusion), though more prominently in the low dose (Barker et al. 2010). Since the conditions under which short, as opposed to long, 22-kHz calls remain unclear at this time, further research should explore which conditions typically evoke various 22-kHz calls. Methodological differences among cocaine administration studies exploring USVs are likely to affect USV emissions and result in inconsistencies between findings. For example, in some experimental designs, USV data are only collected from a subset of the total number of cocaine administration sessions, so daily changes in USVs are not tracked (Barker et al. 2010). In addition, differences in the duration of cocaine access (e.g., short- or long-access) or whether animals are ever removed from the cocaine-paired context would likely influence cocaine-induced or cocaine-conditioned USVs. In the current study, 1-hr cocaine administration sessions occurred 5 days/week, animals

occupied a separate home cage when not participating in training sessions and USVs were recorded during each and every 1-hr conditioning and extinction session. Therefore, the current methodology is unique for the ability to determine the effects of test context, cue abstinence and daily changes in USVs.

Rats emitted more 50-kHz USVs after 2 days of abstinence (i.e., more 50-kHz USVs on Mondays vs. the previous Fridays) during conditioning and during extinction training. Drug deprivation-type effects have been reported not only for cocaine (Grimm et al. 2001; Kerstetter et al. 2008; Lu et al. 2004; Maier et al. 2010; Tran-Nguyen et al. 1998) but also for alcohol and nicotine (Bell et al. 2006; O'Dell and Koob 2007; Rodd et al. 2004; Sinclair and Senter 1968). However, in contrast to the cocaine deprivation effect described for anticipatory 50-kHz USVs (Maier et al. 2010), which is evident even at the Monday following the first weekend deprivation, a marked increase in alcohol consumption per se has so far been shown to occur only with many weeks of extended experience or long periods of abstinence (Bell et al. 2006; O'Dell and Koob 2007; Rodd et al. 2004; Sinclair and Senter 1968). Likewise, in the present report only the number of 50-kHz USVs, but not the amount of horizontal locomotion and reinforced or non-reinforced lever responses, was significantly enhanced after weekends. Since the 2-days of abstinence occurred only on weekends, it is possible that non-specific events occurring only on these particular days may alter behaviors observed on Mondays. Since only USVs and not locomotor activity or lever responding were significantly altered, USVs could be more sensitive to these non-specific events. Previous “incubation of cocaine seeking” studies report that a minimum of 7 or more abstinence days is required to produce increased lever response behavior during extinction training (Grimm et al. 2001; Kerstetter et al. 2008; Lu et al. 2004; Tran-Nguyen et al. 1998). Therefore, our data might indicate that a 2-day drug deprivation period was not long enough to affect lever response

rates, but was long enough to enhance events that mediate USVs. USVs could be a more sensitive measurement of learned associations between the environment and cocaine delivery (i.e., "cocaine expectation") than lever response behavior. Sensitization of drug-induced 50-kHz USVs after repeated same-dose drug exposure has been previously shown for short-term cocaine and amphetamine treatment (Ahrens et al. 2009; Mu et al. 2009; Williams and Undieh 2010). Thus, the weekly enhancement of cocaine-induced 50-kHz USVs on Mondays (e.g., after 2 days of total cocaine and context abstinence) during conditioning might be the result of USV-specific incubation effects since lever responding for drug intake does not yet differ between those days. Alternatively, the Monday boost may reflect a transiently slight degradation of conditioned tolerance to the drug due to forced abstinence away from cues, context and/or drug. During extinction, the elevation of 50-kHz calls on Mondays (e.g., after 2 days of context abstinence) might be triggered by the presentation of direct cocaine-paired cues, such as returning to the operant environment, lever presentation and/or the illumination of stimulus lights. This finding is consistent with previous work reporting animals that previously received amphetamine, morphine or cocaine for several days in a distinct environment, vocalize more in a drug-free state in the drug-paired environment (Knutson et al. 1999; Ma et al. 2010; Maier et al. 2010).

In summary, increasing levels of cocaine experience resulted in higher response rates for SA cocaine rats. Interestingly, the number of FM 50-kHz USVs per cocaine injection declined after approximately two weeks of cocaine sessions. During extinction, USVs dropped off rapidly compared to lever responding. In addition, 2-day periods of abstinence from cocaine and/or cocaine cues exaggerated USVs, but not drug consumption or non-reinforced lever responding. The data support the possibility that

USVs can provide meaningful information in cocaine-experienced rats that cannot be accessed using only lever pressing or locomotor activity behaviors.

Chapter 6: Conclusions

Up to today, there is no effective treatment for cocaine dependence. Besides the fight to overcome drug seeking and wanting, drug dependents have to deal with drug associations that interfere greatly with drug effect and their drug intake behaviors. My research provides additional evidence with respect to earlier studies recognizing the significance of drug associations in the development and persistence of cocaine dependence. New treatment approaches must be identified to overcome strong drug associations. USVs are an essential tool to investigate drug effects and drug associations. In contrast to common measures in drug dependence studies, such as locomotor activity, place preference, drug self-administration, and other invasive brain methodologies, USVs open the horizon to non-invasive and real-time measurements of the affective states of rats in any state of drug use, abuse and dependence.

This dissertation explains the importance of cocaine-induced emotional effects as well as cocaine associations in drug taking behavior. In addition, my dissertation work leads to approaches to find novel treatment interventions.

Firstly, I explored the effects of the anxiolytic drug diazepam on cocaine self-administration behavior and showed that in addition to cause a faster administration of the first cocaine dose, diazepam pretreatment leads to a higher cocaine intake during the sessions. The results from this study indicate that cocaine self-administration behavior is influenced by enhanced GABA activation. Co-administration of sedatives like diazepam seem to alter the incentive to administer cocaine; in the way that there is less hesitation to obtain the first cocaine dose. Importantly, cocaine abusers do not solely take one cocaine dose; the first dose generally initiates binges of drug taking. Therefore, co-administration

of sedatives increases the likelihood of cocaine binges since physiological, protective inhibition processes to administer the first drug dose are attenuated. In addition, cocaine abusers that co-administer sedative drugs might also be more vulnerable to excessive drug abuse due to other alternations of cocaine effects. For example, high doses of cocaine can lead to panic attacks and paranoia, which normally results in a reduction in cocaine intake. Sedatives may mask these aversive effects, which consequently results in excessive cocaine intake. Correspondingly, therapeutic interventions that maximize the aversive effects of cocaine may be a novel means of decreasing cocaine abuse. Therefore, treatment alternatives could consist of the administration of low doses of GABA inverse agonists or CRH agonists to enhance the aversive effects of cocaine without eliciting their own effects. This could be accomplished by the administration of for example slow-releasing implants or injections to achieve steady drug concentrations.

Secondly, I investigated the impact of long-term cocaine administration as well as environmental stimuli associated with cocaine intake on cocaine-induced behaviors, such as locomotor activity, lever responding and USVs. In addition, I studied how these behaviors change during extinction training and if these three measures correspond with each other throughout the experiment.

My research revealed that environments associated with cocaine intake progressively enhance the emission of 50-kHz USVs (i.e., positive affect). In contrast, cocaine-induced positive USVs diminish while cocaine intake increases (i.e., decrease in USV/lever response ratio). The interpretation of these findings gives two possible explanations: 1) The previously neutral but now cocaine-paired environment acts as a conditioned stimulus and elicits a conditioned response. The decrease in cocaine-induced 50-kHz USVs implies that the response to the actual cocaine reward shifts to the preceding conditioned stimulus. 2) Increased emission of 50-kHz USVs in the drug-

paired environment reflects anticipatory calling/wanting for cocaine. Locomotor activity supports the increased expectation of impending cocaine administration, since the animals show increased activity in the drug-paired environment, indicating possibly excitement, anticipation and cocaine seeking. In addition, after 2-day abstinence, environment-induced USVs increase by approx. 70%, reflecting a greater anticipation of or wanting for cocaine administrations. This is of particular interest since cocaine-associated stimuli have been reported to initiate cocaine-seeking and relapse in abstinent cocaine users. Pharmacological interventions disrupting cue- or environment-induced cocaine cravings are from major importance in the fight against cocaine dependence and will be discussed later. 3) Furthermore, the decreased ability of cocaine to induce positive affect during long-term cocaine administration might reflect the development of tolerance-like effects to the rewarding effects of cocaine. Importantly, after short-term (i.e., 2-day) abstinence to cocaine and the cocaine-paired environment, cocaine-induced 50-kHz USVs, but not drug intake are elevated compared to the previous session. This increase of cocaine-induced positive affect after a 2-day abstinence supports the development of drug tolerance-like effects to cocaine reward. Tolerance is also a major factor that contributes to the manifestation of compulsive drug intake and the development of cocaine dependence in human drug abusers, since more cocaine is needed to compensate for reduced cocaine effects. From these findings, alternative interventions could be included in psychotherapies, such as incorporation of phases of abstinence to regain drug reward. This intervention would not lead to a cessation of cocaine intake, but consequently reduce overall cocaine intake. Reduction in cocaine intake results in less frequent allostatic states, less cocaine-induced hospitalizations, more drug-free days and less drug procurement costs including less procurement crime, all of which results in enhanced opportunities to participate in social activities. The anticipation

of enhanced cocaine reward, lower cost, and more "normal life" might even be an incentive for staying abstinent.

Environment-induced 50-kHz USVs extinguish over the course of extinction, but are more persistent in animals that do not operate the lever (i.e., receive yoked non-reinforced injections). This indicates that self-administration of non-reinforced injections during extinction training attenuates cocaine seeking more effectively than yoked administration. The loss over previous control over cocaine consumption (i.e., lever responses were reinforced) leads to a decrease in cocaine expectation and consequently effort to obtain cocaine (i.e., reduction in lever responses). As a result, the disruption of strong cocaine-paired associations occur faster. Therefore, therapeutic intervention should include the active handling of cocaine paraphernalia involving self-administration of non-psychoactive substances (e.g., finely milled sodium chloride), preferably in the individual's cocaine-paired context. For example, therapeutic intervention could take place in a room filled with personal cocaine paraphernalia, and the subject is offered to consume as much non-psychoactive substance as wanted. Eventually, the expectation of cocaine consumption elicited by the paraphernalia will attenuate. This finding also emphasizes the importance of the use of the self-administration paradigm for drug dependence research.

Conditioned 50-kHz USVs are enhanced after 2-day abstinences, indicating that even the smallest break from extinction training can lead to recurrence of extinguished behaviors. In addition, lever responding does not extinguish completely for the duration of the experiment. These findings lead to two plausible explanations: 1) Animals operate the lever despite unexpected cocaine delivery (i.e., environment-induced USVs quickly extinguish) and "check" for drug availability or 2) previous operant training for cocaine became habitual or automated due to underlying neuroadaptive changes caused by

frequent cocaine abuse. In any of these two cases, a therapy involving cocaine paraphernalia handling and self-administration of non-psychoactive powder/injections seems very promising. Since extinguished behaviors, such as environment-induced locomotor activity and 50-kHz USVs increase dramatically after 2-day abstinences (approx. 20 or 100%, respectively), this therapy shows resistance after short periods of therapy interruptions (i.e., 2 days) and has to be continuously. In addition, pharmacological therapy of memory-enhancing drugs during extinction training might greatly support this intervention. This assumes that rather than "unlearning" strong cocaine associations and habits, "disruption" of these phenomena occurs through a new learning process.

My findings show that USVs do not only support current findings, they reveal effects that are not detected with common measures such as locomotor activity and drug self-administration. For example, despite increased cocaine intake, cocaine's rewarding effects decrease after only two weeks of cocaine experience (i.e., discovery of tolerance to the rewarding effects of cocaine). In addition, conditioned anticipation for impending drug administration is reflected by increased locomotor activity and 50-kHz USVs. However, during extinction training, only the measurement of 50-kHz USVs detects non-extinguished anticipation for cocaine. Furthermore, after 2-day abstinences throughout conditioning only USV measurements discover the enhancement of rewarding effects of cocaine (approx. 100% increase). Following 2-day abstinences during extinction training, intensification of cocaine craving is detected by locomotor activity (approx. 20% increase), but more intense craving is revealed through 50-kHz USVs (approx. 100% increase). Since food-conditioned USVs (i.e., USVs emitted on day 1 prior to the first cocaine exposure) are correlated with reward magnitude of early and late cocaine

experience (i.e., USV/lever response ratio), food-conditioned USVs could be a selection tool for animals experiencing and maintaining positive cocaine-induced affect.

These findings support the notion that USVs are an important additional measurement for drug dependence (and other animal) studies, since they do not only give insight into the affective states of the animals, they also seem to be more sensitive compared to common measures. Therefore, USVs may provide a pre-clinical model of drug craving and drug reward that will help to develop treatments to block or interfere with the rewarding properties of drugs of abuse, impulsive drug taking, drug associations, and relapse.

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Vita

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